Universidade de Aveiro Departamento de Química
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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Engenharia Química, realizada sob a orientação científica da Doutora Maria Ângela Sousa Dias Alves Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro, da Doutora Luísa Alexandra Seuanes Serafim Martins Leal, Professora Auxiliar do Departamento de Química da Universidade de Aveiro e do Doutor Newton Carlos Marcial Gomes, Investigador Principal do Centro de Estudos do Ambiente e do Mar (CESAM)

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"Within one linear centimeter of your lower colon there lives and works more bacteria than all humans who have ever been born. Yet many people continue to assert that it is we who are in charge of the world."

– Neil deGrasse Tyson

o júri

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Prof. Doutora Maria Ângela Sousa Dias Alves Cunha
professora auxiliar da Universidade de Aveiro

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palavras-chave

Bactérias anaeróbias facultativas, biossurfactante, biodegradação de hidrocarbonetos, sedimentos, mar profundo, estuário da Ria de Aveiro.

resumo

Actualmente são conhecidas poucas estirpes bacterianas capazes de produzir biossurfactantes (BSFs) em condições de microaerobiose ou anaerobiose. Estas bactérias têm um papel importante não só em processos naturais (ex. formação de biofilmes ou de hidratos de gás), como podem ter diversas aplicações biotecnológicas (ex. estratégias de biorremediação e aplicações industriais). As bactérias produtoras de BSFs em condições de limitação de oxigénio, com capacidade para degradar hidrocarbonetos são de particular interesse para estratégias de biorremediação de locais contaminados com hidrocarbonetos de petróleo (PHs) e na recuperação microbiana de petróleo (MEOR). Neste contexto, o objectivo deste trabalho foi o isolamento, identificação e a caracterização de bactérias anaeróbias ou anaeróbias facultativas produtoras de BSF e degradadoras de hidrocarbonetos (hidrocarbonoclásticas) na perspetiva da sua aplicação biotecnológica em condições de limitação de oxigénio.

Foram escolhidos dois ambientes contaminados com PHs como potenciais fontes de bactérias hidrocarbonoclásticas produtoras de BSFs: vulcões de lama (MV) de mar profundo do Golfo de Cádis (Oceano Atlântico) e o sistema estuarino da Ria de Aveiro (Portugal). Foram preparadas culturas de enriquecimento com sedimentos subaquáticos recolhidos nestes dois habitats, como potenciais inóculos de bactérias anaeróbias facultativas. Um design experimental fatorial foi usado para testar o efeito do crude como fonte de carbono, e de nitrato e/ou sulfato, como aceitadores terminais de eletrões.

De forma a melhor compreender a estrutura das comunidades bacterianas envolvidas na biodegradação de PHs nos MV do mar profundo procedeu-se à sequenciação do gene 16S rRNA das comunidades bacterianas de culturas de enriquecimento com sedimento de dois MVs, um activo e outro inactivo, e com ou sem adição de crude e/ou nitrato. Detetou-se uma diferenciação entre as comunidades dos dois MVs, independentemente dos suplementos a que as culturas foram expostas, sendo que Alphaproteobacteria e Bacilli predominaram nas culturas com sedimentos de MV activo e inactivo, respectivamente. De uma forma menos acentuada, tanto o nitrato como o crude afetaram a composição das comunidades bacterianas. Géneros de bactérias que só foram detectados nos ensaios com adição de crude (ex. Erythrobacteraceae no MV activo e Acidimicrobiale no MV inactivo) poderão ser usados como indicadores da presença de hidrocarbonetos de petróleo nestes habitats.

resumo (cont.)

A biodegradação de PHs nas culturas com crude foi avaliada por cromatografia gasosa acoplada a espectrometria de massa. De uma forma geral, as comunidades de culturas do MV activo foram capazes de degradar *n*-alcanos de tamanho inferior a C_{13} e compostos monoaromáticos, enquanto as comunidades do MV inactivo apresentaram a capacidade de metabolizar vários tipos de hidrocarbonetos aromáticos policíclicos. A presença de nitrato apenas afectou positivamente a biodegradação de alcanos, e não teve efeito ou foi mesmo inibitória da biodegradação de outros hidrocarbonetos.

A partir de todas as culturas, com todos os tipos de sedimentos, dos MVs do Golfo de Cádis e do estuário da Ria de Aveiro, foi possível isolar-se um total de 13 isolados capazes de sobreviver exclusivamente com crude como fonte de carbono e produzir BSF em condições de aerobiose. Destas, apenas duas não foram capazes de produzir BSFs em anaerobiose. A sequenciação do gene 16S rRNA dos isolados permitiu identifica-los como pertencendo aos géneros *Pseudomonas*, *Bacillus*, *Ochrobactrum*, *Brevundimonas*, *Psychrobacter*, *Staphylococcus*, *Marinobacter* e *Curtobacterium*, a maioria dos quais não tinha ainda membros conhecidos como produtores de BSF em anaerobiose.

Os resultados obtidos com este trabalho permitiram caracterizar melhor as comunidades envolvidas na degradação de PHs em MVs de mar profundo. Conseguiu-se ainda isolar e identificar estirpes, tanto de mar profundo como de ambiente estuarino, capazes de degradar PHs e produzir BSFs em condições de anaerobiose. Estas estirpes apresentam elevado potencial biotecnológico para aplicações como MEOR e biorremediação em ambientes com escassez de oxigénio.

keywords

Facultative anerobic bacteria, biosurfactant, biodegradation of hydrocarbons, sediments, deep-sea, Ria de Aveiro estuary.

abstract

So far, only few bacterial strains are known to produce biosurfactants (BSFs) under microaerobic or anaerobic conditions. However, these bacteria are not only involved in important natural processes (e.g. biofilm and gas hydrates formations) but can also be used in several biotechnological applications (e.g. bioremediation strategies and industrial applications). Bacteria able to produce BSFs under oxygen-limiting conditions that are also able to degrade hydrocarbons, are of particular interest to bioremediation strategies of sites contaminated with petroleum hydrocarbons (PHs) and microbial enhanced oil recovery (MEOR) strategies. In this context, this work aims at isolating, identifying, and characterizing BSF-producing and hydrocarbon-degrading (hydrocarbonoclastic) bacteria grown under anaerobic conditions, which can be used in biotechnological applications under oxygen limitation.

Two environments contaminated with PHs were chosen as potential sources of hydrocarbonoclastic BSF-producing bacteria: deep-sea mud volcanos from the Gulf of Cadiz (Atlantic Ocean), and the estuarine system of Ria de Aveiro (Portugal). Enrichment cultures were prepared using subaquatic sediments from both sites, as potential sources of facultative anaerobic bacteria. A factorial experimental design was used to test the effect of crude oil as carbon source, and nitrate and/or sulfate, as terminal electron acceptors.

Aiming at better understanding the structure of bacterial communities involved in PHs biodegradation at deep-sea MVs, sequencing of the 16S rRNA gene was performed for bacterial communities from cultures containing sediments from two MVs, active and inactive, and with or without crude oil and/or nitrate. A distinction between the communities of MVs with different activity, independent of the supplements was observed. Alphaproteobacteria and Bacilli were the predominant classes found in enrichment cultures inoculated with active and inactive MVs sediments, respectively. In a minor scale, nitrate and crude oil additions also affected the composition of bacterial communities. Therefore, genera that only appeared in cultures with crude oil. (e.g. Erythrobacteraceae in active MV cultures and Acidimicrobiale in inactive MV cultures) can be used as biosensors of the presence of PHs in these habitats. Biodegradation of PHs in cultures containing crude oil was assessed by gas chromatography coupled with mass spectrometry. Overall, communities from active MV cultures were able to degrade *n*-alkanes below C_{13} and monoaromatic hydrocarbons, while communities from inactive MV cultures presented the ability to metabolize several types of polycyclic aromatic hydrocarbons. The presence of nitrate only had a positive effect on the biodegradation of alkanes, and had no effect or even an inhibitory effect on the biodegradation of other hydrocarbons.

abstract (cont.)

A total of 13 isolates able to survive on crude as carbon source and produce BSF under aerobic conditions were obtained from all cultures either from sediments of the Gulf of Cadiz MVs or the estuarine system of Ria de Aveiro. Only two isolates failed to produce BSF under anaerobiosis. Sequencing of 16S rRNA gene was used to establish the identification of isolates as *Pseudomonas*, *Bacillus*, *Ochrobactrum*, *Brevundimonas*, *Psychrobacter*, *Staphylococcus*, *Marinobacter* and *Curtobacterium*. Most of these genera had never been described as able to produce BSFs under anaerobic conditions.

The results obtained in this work allowed to better characterize the deep-sea communities involved in PHs degradation, as well as, to identify strains from deep-sea and estuarine sediments able to degrade PHs and produce BSFs under anaerobic conditions. These bacteria present high biotechnological potential for applications in oxygen-limiting environments, such as, MEOR and bioremediation of environments contaminated with PHs.

CONTENTS

Abbreviations

BSF	Biosurfactant
CMC	Critical micellar concentration
COD	Chemical oxygen demand
DGGE	Denaturing gradient gel electrophoresis
DWH	Deepwater Horizon
E_{24}	Emulsification index after 24 h
EOR	Enhanced oil recovery
FeRB	Fe(III)-reducing bacteria
GC-MS	Gas chromatography–mass spectrometry
HPLC	High-pressure liquid chromatography
LA/B	Luria Agar/Broth
MAH	Monoaromatic hydrocarbon
MB2216	Marine Broth 2216
MEOR	Microbial enhanced oil recovery
MSM	Mineral salts medium
MV	Mud volcano
NRB	Nitrate-reducing bacteria
OTU	Operational taxonomic unit
PAH	Polycyclic aromatic hydrocarbon
PCO	Principal coordinates analysis
PH	Petroleum hydrocarbon
SRB	Sulfate-reducing bacteria
TEA	Terminal electron acceptor

List of figures

List of tables

THESIS OUTLINE AND OBJECTIVES

Biodegradation of petroleum hydrocarbons (PHs) is a natural phenomenon that naturally occurs in environments where petroleum or its by-products are present, such as petroleum reservoirs or sites contaminated with petroleum due to anthropogenic actions. In many cases, hydrocarbon biodegradation is due to bacterial activity. These hydrocarbonoclastic bacteria have a high biotechnological potential. They are mainly involved in microbial enhanced oil recovery (MEOR) and bioremediation strategies. In both applications, the production of biosurfactants (BSFs), molecules that decrease the interfacial tension, can be beneficial and improve the success rate of these strategies. Bacterial BSF-production by itself is of biotechnological interest, with most BSFs being more stable, biodegradable and less toxic than synthetic surfactants. BSFs are used in several industries such as food and pharmaceutical. However, so far there is little variety of known BSFs and few of them are produced industrially. As such, new bacterial strains, especially those isolated from extreme environments, may help increase the known BSF pool and their applications.

Most of the known hydrocarbonoclastic and BSF-producing bacteria were isolated under aerobic conditions, even if their original environment lacked oxygen. The study of anaerobic bacteria is often more time consuming and demanding, from a materials and methods point of view, than the study of aerobic bacteria. As a consequence, anaerobic bacteria are less studied than their aerobic counterparts. None the less, hydrocarbonoclastic and/or BSF-producing bacteria functioning under oxygen-limiting conditions play important ecological roles in environments (e.g. altering petroleum composition and in formation of gas hydrates) and biotechnological applications (e.g. MEOR and bioremediation strategies in locations lacking oxygen).

Therefore, the main objective of this work is to increase the current knowledge regarding hydrocarbonoclastic BSF-producing facultative anaerobic bacteria with biotechnological potential. More specifically, this work aims at identifying key players involved in anaerobic PHs biodegradation in gas seepage sites, such as the Gulf of Cadiz mud volcanos (MVs), to better understand the hydrocarbon degradation capability of anaerobic communities from these environments and their response to crude oil, as a carbon source, and nitrate, as a possible terminal electron acceptor (TEA). Furthermore, this work aims at isolating, identifying and characterizing hydrocarbonoclastic BSF-producing facultative anaerobic bacteria originated from subaquatic sediments from the deep-sea collected at MVs of Gulf of Cadiz and the estuarine system of Ria de Aveiro.

Chapter 1 reviews the theme of bacterial BSF-production under oxygen-limiting conditions. Known anaerobic or microaerobic BSF-producing bacteria are discussed, as well as, their metabolic pathways involved in BSF production, growth factors affecting BSF production and applications.

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Chapter 2 summarizes the state-of-art on anaerobic hydrocarbonoclastic bacteria, their applications in bioremediation strategies and subaquatic sediments as potential sources for their isolation.

Chapter 3 describes the preparation of enrichment cultures containing sediments from active and inactive deep-sea MVs, as inoculum, presence or absence of crude oil, as sole carbon source, and nitrate, as possible TEA. The composition of bacterial communities was analyzed high-throughput 16S rRNA gene sequencing. A major trend observed was the differentiation in most sub-samples regarding origin of sediment. Treatments only had minor impacts on the structure of bacterial communities, with some genera only being present in the presence of crude oil. Crude oil biodegradation was assessed for the enrichment cultures containing crude oil. The results indicate that communities from active MVs were better adapted at degrading *n*-alkanes and monoaromatic PHs, while communities from inactive MVs showed higher ability at degrading polycyclic aromatic hydrocarbons.

Chapter 4 reports on the effort to isolate and characterize new facultative anaerobic hydrocarbonoclastic BSF-producing bacteria. Anaerobic selective cultures prepared from marine sediments from deep-sea MVs or the estuarine subtidal sediments from Ria de Aveiro were prepared. A factorial experiment design was followed to test the effect of, crude oil, as carbon source, and nitrate and/or sulfate, as TEAs. Through successive cultures in solid media incubated under aerobiosis, 13 isolates able to produce BSF were obtained. Of these, 11 grew and produced BSF under anaerobic conditions. The isolates were identified as belonging to several genera not known to contain members able to produce BSF under anaerobic conditions.

Chapter 5 summarizes the main results obtained with this work, drawing conclusions and indicating future perspectives for both further characterization of the isolates identified, as well as, their application.

Chapter 1

CHAPTER 1

Bacterial production of biosurfactants under microaerobic and anaerobic conditions

Patrícia M. Domingues, Adelaide Almeida, Luísa S. Serafim, Newton C. M. Gomes, Ângela Cunha

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1.1. Abstract

Almost all knowledge about bacterial production of biosurfactants (BSFs) is limited to aerobic conditions. However, it is also known that bacteria can produce BSFs under oxygen-limiting conditions. These substances may be involved in important environmental processes (e.g. formation of gas hydrates and biofilms) or be applied in biotechnological processes (e.g. bioremediation and microbial enhancement of oil recovery, MEOR). Up to now, only few bacteria are described with the ability to produce BSFs under microaerobic and anaerobic conditions. Most of them belong to the *Bacillus* and *Pseudomonas* genera. However, BSF production under oxygen limitation has been detected in other bacterial groups (e.g. *Anaerophaga* and *Thermoanaerobacter*) involving different biosynthetic pathways. In this review, we summarize the current knowledge on growth requirements, cultivation conditions and properties of BSFs produced under oxygen-limiting conditions. In addition, we discuss the potential applications of microaerophilic and anaerobic BSF-producing bacteria in the perspective of bioremediation or MEOR strategies, energy and industry.

1.2. Key words

Anoxia, *Bacillus mojavensis* JF-2, microbial enhanced oil recovery, bioremediation, gas hydrates.

1.3. Introduction

Surfactants are amphiphilic molecules composed of both hydrophobic and hydrophilic moieties. This allows them to concentrate at interfaces between phases and modify them in order to promote dispersion of one phase into the other. Surfactants are able to form aggregate structures, such as micelles, bilayers and vesicles[1]. Critical micellar concentration (CMC) is the minimum concentration in which surfactants in solution are able to spontaneously form micelles. Therefore this parameter is useful for comparing different surfactants, their activity and efficiency[2]. Surfactants with lower CMC values are considered to be more efficient since a smaller amount of surfactant is needed to decrease the surface tension[3]. In instances where surfactants are also able to produce

emulsions, these compounds are also called emulsifiers[4] as they induce the formation of emulsions, that is, the dispersion of one phase in another in the form of droplets[5].

Biosurfactants (BSFs) are molecules with identical properties to surfactants but produced by living organisms, such as bacteria. In microorganisms, BSFs are naturally involved in microbial competitive interaction[6], cell-to-cell communications[7], plant and animal pathogenesis[8], increasing the bioavailability of surface-bound subtracts and heavy metals (via direct interfacial contact and pseudosolubilization)[9–12], avoidance of toxic elements and compounds[13,14], motility[15] and biofilm formation and maintenance[8,16–18]. Taking advantage of some of these characteristics, under biotechnological and industrial contexts, BSFs are used mainly as antimicrobial agents, detergents, emulsifiers, dispersants and foaming or wetting agents[19,20]. Their industrial applications are related to the pharmaceutical industry and production of cosmetics and personal care products, biological pest control and agriculture, food industry, bioremediation and microbial enhanced oil recovery (MEOR), mining and metallurgical industries[19–22].

BSFs are particularly advantageous when compared to synthetic surfactants due to lower toxicity and higher biodegradability[23,24]. These are the main reasons why they are preferred over synthetic surfactants in bioremediation and industrial applications. In general, BSFs also present better emulsifying properties and good stability at extreme pH, salinity and temperature values[25–27]. However BSF production and competitive commercialization is still hampered by higher production costs, lower variety of produced BSFs and inefficient transfer of scientific knowledge to the industry[22].

Production of BSF in oxygen-limiting conditions occurs naturally in environmental conditions, such as during gas hydrates formation[28], and can have application in biotechnological processes which may require the production of BSF in environments poor in oxygen, like MEOR[29]. However, potential BSF-producing bacteria isolated from anaerobic or microaerobic environments, such as oil wells and sediments, are often only grown and tested for production of BSF under aerobic conditions due to time and material restrictions[20,30–33]. In the context of this review, an environment will be considered as aerobic when molecular oxygen (O_2) corresponds to 21% or more of gaseous environments or there is over 30% oxygen saturation in aquatic environments. Microaerobiosis correspond to lower percentages of O_2 than in aerobic conditions, 1-30% of oxygen saturation in liquid media and less than 21% in air. Strict anaerobiosis is the complete lack of O_2. Microbes can be classified depending on their ability to grow under these conditions: obligate aerobes require O_2 for growth; facultative anaerobes can use several terminal electron acceptors (TEAs) including O_2 or operate fermentative pathways; microaerophiles can use O_2 but only in lower concentrations than those present in normal atmosphere/solutions, usually 1-15% in air or less than 30% saturation of

oxygen in liquid media; aerotolerant anaerobes do not use O_2 as a TEA but can live in its presence; and strict or obligate anaerobes not only do not use O_2 but are also inhibited or even killed in its presence[34,35].

BSF-producing bacteria have been extensively reported in scientific literature, especially under aerobic conditions, and several detailed reviews about the topic have already been published[3,36]. However, relatively few studies addressed microaerobic and anaerobic conditions, even though the earliest mention of production of BSF under anaerobiosis was published in 1955[37]. Since then few bacteria have been identified as being able to produce BSF under oxygen-limiting conditions, with most of these being described after the turn of the millennia. The present review intends to summarize and systematize the current knowledge on production of bacterial BSF under microaerobic and anaerobic conditions thus contributing to an in depth perspective on ecology of BSF-producing bacteria and their potential biotechnological and industrial applications.

1.4. Anaerobic and microaerobic biosurfactant-production: players and products

Few bacteria are known to produce BSFs under oxygen-limiting conditions. In this section, these bacteria are grouped by genera and relevant characteristics and the BSFs produced will be highlighted. *Bacillus mojavensis* JF-2, which is the most well studied anaerobic BSF producer, will be addressed separately as a biological model for anaerobic BSF production. A summary of bacterial isolates known to produce BSF in oxygen-limiting conditions can be found in Table 1.1 and respective culturing information in Table 1.2. Table 1.3 lists known properties of BSFs produced under microaerobic and anaerobic conditions. All tables can be found in section 1.10.

1.4.1. *Bacillus mojavensis* JF-2

B. mojavensis JF-2, previously known as *Bacillus licheniformis* JF-2[38] is able to produce the same type of surface active molecule, referred as BSF JF-2 or lichenysin B[39,40], under aerobic and anaerobic conditions. In both conditions, it lowers the surface tension of media by approximately 40 mN/m[41]. In anaerobiosis, B. *mojavensis* JF-2 uses nitrate as primary TEA. Anaerobic cultures grow slower than aerobic ones but, in both cases, the BSF is produced and released during exponential phase of growth[41], being rapidly assimilated by B. *mojavensis* JF-2 cells at the beginning of the stationary phase[42]. Uptake of BSF by cells during stationary phase is mediated by unknown cell surface components[42]. The internalized BSF is thought not to be used as carbon or energy source and the uptake process is inhibited by magnesium ions[42]. It has been hypothesized that the internalization of the BSF may be connected to a change in the development of the bacteria, possibly working as a chemical signal[42]. Due to the narrow window of BSF production before it is taken up by stationary phase cells and because of the loss of ability to produce BSF that occurs after several generations in

liquid cultures[41], large scale production of BSF by *B. mojavensis* JF-2 still presents major technical challenges. Although regarded as promising for MEOR strategies, *B. mojavensis* JF-2 is not particularly appealing for large scale BSF production, in comparison with other microorganisms with less complex BSF formation kinetics.

Lichenysin B is an anionic BSF with a molecular weight of 1035 Da and *B. mojavensis* JF-2 produces only one isoform[43]. Lichenysins are structurally very similar to surfactin (Figure 1.1) and are sometimes classified as an isoform of the later[44]. Lichenysin B is a cyclic lipopeptide, composed of a heptapeptide structurally identical to surfactin (L-Glu – L-Leu – D-Leu – L-Val – L-Asp – D–Leu – L–Leu), with a β-hydroxy fatty acid amidated to the N-terminal amine of the peptide, while the C-terminal amino acid of the peptide is esterified to the β-hydroxy group of the fatty acid, forming a lactone ring (Figure 1.1)[43,45,46]. The β-hydroxy group is mainly composed of either a normal, *iso*, or *anteiso* branched C_{14} or an *iso* or *anteiso* C_{15} [39]. This results in a 'horse saddle' conformation, which is mostly responsible for the properties of the molecule, such as its antimicrobial activity[47,48]. Both NaCl and Ca concentrations affect lichenysin B effectiveness. NaCl concentrations above 50 g/L are required to lower interfacial tension (< 0.1 mN/m) and calcium concentrations above 25 g/L lead to an increase in surface tension (> 2 mN/m), independently of temperature (Table 1.3, section 1.10)[49]. Lichenysin B presents a CMC of 10 mg/L[43], which makes it effective even in small concentrations and able to reduce interfacial tensions to very low values (< 0.1 mN/m)[49]. Lichenysin B is stable when exposed to 25-120 °C for 20 min[40] and exhibits immunological and biochemical responses similar to surfactin, due to its almost identical hydrophilic moiety[43]. However, while surfactin is unstable in the presence of NaCl, lichenysin B is active in concentrations of up to 100 g/L[40].

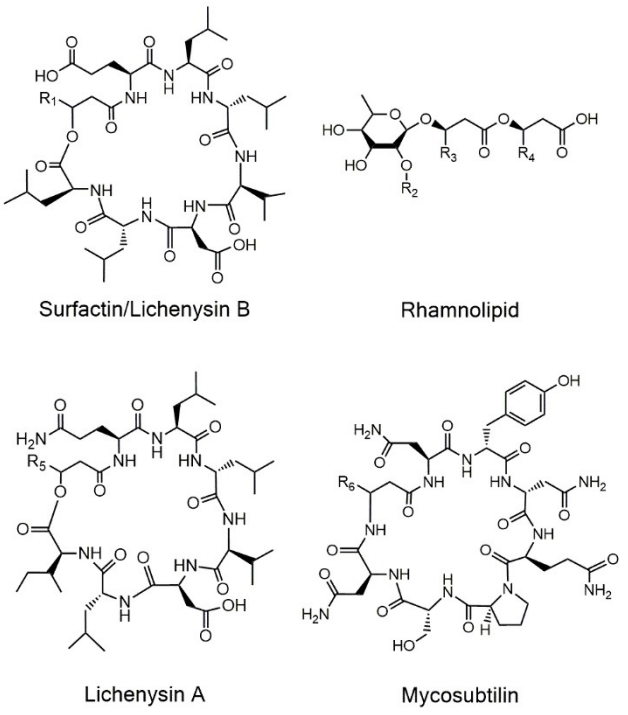

Figure 1.1 – Generic chemical structures of main BSFs produced in oxygen-limited conditions by bacteria. R_1: C_{12}-C_{16} β-hydroxy fatty acid in surfactin and C_{12}- C_{17} in lichenysin B[39]. R_2: a hydrogen atom or α-L-rhamnopyranosyl. R_3 and R_4: C_8-C_{16} β-hydroxy fatty acid[523,524]. R_5: C_{12}-C_{17} β-hydroxy fatty acid[39]. R_6: C_{15}-$_{17}$ β-hydroxy fatty acid[58].

1.4.2. Other *Bacillus* species

With the discovery and characterization of lichenysin B, close relatives of *B. mojavensis* JF-2 were soon screened for similar BSFs. Most studies were conducted in the presence of O_2 but the

production of an analogue molecule, lichenysin A, was detected in *B. licheniformis* BAS50 cultivated in anaerobic conditions[39]. *B. licheniformis* BAS50 is a facultative anaerobe isolated from an oil reservoir at 1500 m of depth, capable of reducing nitrate and producing spores only under aerobic conditions. Similarly to *B. mojavensis* JF-2, aerobic growth of *B. licheniformis* BAS50 resulted in a shorter lag phase, higher biomass production and lower surface tension than anaerobic cultivation, with minimum values of surface tension of 28.3 mN/m and 35 mN/m for aerobic and anaerobic conditions, respectively. In both conditions, the stationary phase was achieved within the same time. BSF, identified as lichenysin A, was produced throughout the exponential phase in aerobic and anaerobic conditions. Lichenysin A is structurally very similar to lichenysin B (Figure 1.1), differing in the amino acid sequence, Glx-Leu-Leu-Val-Asx-Leu-Ile, and the β-hydroxy fatty acid which ranges from C_{12} to C_{17} normal, *iso* and *anteiso* forms, with the most common being *iso* C_{15} (38.6%). Additionally, lichenysin A has antimicrobial properties, albeit being less effective than surfactin[39].

Other nitrate-reducing strains (nitrate-reducing bacteria, NRB) of *B. licheniformis*, namely strains BNP29, BNP36 and Mep132 isolated from the same oil reservoir as *B. licheniformis* BAS50, were also able to produce BSFs under anaerobic conditions[50]. Although less productive than *B. licheniformis* BAS50 (Table 1.1, section 1.10), they were also able to produce an extracellular polymer. When cultivated in anaerobic conditions, strains BNP29, BNP36 and Mep132 displayed a longer lag phase and shorter exponential and stationary phases. BSF production was identical to the *B. licheniformis* BAS50, and all strains produced larger amounts of BSF and smaller amounts of polymer in aerobic conditions, in comparison to anaerobiosis[50]. The linkage between the biochemical pathways of BSF and polymer is still not understood but is possible that the lower yield of BSF in anaerobic conditions is linked to a diversion of carbon to biosynthetic pathways of polymer production.

In a screening of isolates with MEOR potential, *B. mojavensis* and *B. licheniformis* strains able to produce BSFs under anaerobic conditions in the presence of 50 g/L NaCl were detected[46]. In later studies testing the feasibility of a MEOR strategy, *Bacillus* RS-1 and *Bacillus subtilis* subsp. *spizizenii* NRRL B-23049, both NRB, were able to produce BSF under anaerobic conditions, and even in the oil well environment[51,52]. Although oxygenation conditions inside the wells were not described, they are expected to correspond to a microaerobic or anaerobic environment[29]. Oil production was stopped for 108 h after bacteria inoculation and addition of nutrients. The average concentration of BSF in the inoculated oil wells when pumping was restarted was 90 mg/L, and a maximum of 350 mg/L was reached at approximately 10 h of pumping. In the inoculated oil wells, other non-spore forming bacteria, supposedly originated from the non-sterilized tanks where inoculum and nutrients were mixed, were able to produce BSF when a nutritional supplement, composed of glucose and mineral salts, was added to the wells. However, after 108 h of incubation

in the oil wells, only the two inoculated *Bacillus* strains were recovered and confirmed as still able to produce BSF. Most metabolites detected in inoculated wells after 140 h of incubation were related to the anaerobic use of glucose. In laboratory experiments under anaerobic conditions a consortium of both *Bacillus* strains presented a growth rate of 0.18 h^{-1} and BSF yield of 0.02 mol BSF/mol glucose, while in inoculated oil wells, the respective values are 0.06 h^{-1} and 0.02 mol BSF/mol glucose[51]. Thus, while slower growth is observed in *in situ* conditions, BSF yields remain the same as in laboratory conditions.

In a search for strains able to produce BSF in oil reservoir conditions, five *B. subtilis* isolates (#191, #309, #311, #552 and #573) retrieved from crude oil, were tested[26]. All strains were thermo- and halotolerant and able to grow and produce BSFs under both aerobic and anaerobic conditions, by using nitrate as TEA and fermentative pathways. BSFs showed good emulsifying activity, with the ability to reduce the medium surface tension below 40 mN/m (Table 1.1, section 1.10) and presented CMC values between 130 mg/L and 20 mg/L (Table 1.3, section 1.10). BSF produced by strains #309, #311 and #573 in aerobiosis, were identified as isoforms of surfactin with hydroxy fatty acid chains between C_{13} and C_{14} [53].

A comprehensive experimental approach aiming the optimization of BSF production by the NRB *B. licheniformis* VKM B-511 (DSM 13) reported enhanced BSF production yields under anaerobiosis, resulting in an optimized yield of 4.58 g/L. Higher BSF yields and BSFs with higher emulsification indexes and decreased surface activity were obtained in anaerobic cultures when compared to aerobic conditions[54].

Surfactin, one of the more studied and better known BSFs, is a secondary metabolite of the NRB *Bacillus subtilis* ATCC 21332. It is produced since early exponential phase but reaches maximum yields during stationary phase. Studies on the optimization of surfactin production showed that nitrate-limited anaerobic conditions can increase the BSF yield on biomass to 0.075, compared to only 0.021 in aerobic N-limiting conditions[55]. Surfactin production by *B. subtilis* ATCC 21332 under anaerobic conditions is relatively fast, when compared with other bacteria. At 20 °C, BSF can be produced within approximately 24h, which corresponds to the beginning of its exponential phase[28]. Surfactin is structurally very similar to lichenysin B (Figure 1.1), differing in its β-hydroxy fatty acids which can present normal, *iso* or *anteiso* C_{12}-C_{16} fatty acid chains, being *n* C_{14} predominant (40%)[39]. The difference in nomenclature is due to the producing organisms, with lichenysins being produced by *B. licheniformis* and *B. mojavensis* and surfactin being mainly produced by *B. subtilis*[48]. Isoforms of surfactin vary in length or branching of its hydroxy fatty acid moiety[39]. A curious finding is that surfactin produced by *B. subtilis* ATCC 21332 under aerobiosis and anaerobiosis presents the same five isoforms but not in the same proportions[28].

Besides surfactin, *B. subtilis* subsp. *spizizenii* ATCC6633 is able to produce mycosubtilin in microaerobic conditions[56]. Mycosubtilin is a nonribosomal lipopeptide belonging to the iturin family, with known antifungal and surface-active properties. Iturins are composed by a cyclic heptapeptide bonded to a β-amino fatty acid, which chain can vary between C_{15} and C_{17} (Figure 1.1). The main difference between different iturins, including mycosubtilin, is the α amino acid composition of the heptapeptide[57–59]. *B. subtilis* BBG100 is a genetically modified strain of *B. subtilis* ATCC6633 in which the promoter responsible for the mycosubtilin synthetase operon was replaced by a constitutive promoter from Staphylococcus aureus plasmid pUB110. *B. subtilis* BBG100 is able to produce surfactin and mycosubtilin under microaerobiosis, achieving higher mycosubtilin but lower surfactin concentrations than *B. subtilis* ATCC6633 (Table 1.1, section 1.10)[56].

The fermentative *B. subtilis* C9 (KCTC 87 01P) preferred oxygen-limiting condition for the production of BSF C9-BS[60]. BSF production was increased when a 15-hour period of microaerobiosis and anaerobiosis was imposed during the exponential phase in batch fermentation. BSF production started in early exponential phase and decreased during stationary phase. As expected, a shorter lag period was observed in aerobic conditions but biomass and BSF production rates were half and a third, respectively, than in oxygen-limiting condition. Since C9-BS, a lipopeptide-type BSF, is also an emulsifier, it leads to the formation of foam in bioreactors, which is aggravated by the presence of oxygen. Foam overflow causes loss of medium, leading to lower biomass and BSF production. C9-BS presents a CMC of 40 mM and is able to lower the surface tension of water from 72 mN/m to 29 mN/m. Activity is stable in wide ranges of temperature, pH, salinity (Table 1.3, section 1.10) and up to 10 mM of $CaCl_2$ concentration[60].

Bacillus amyloliquefaciens S499, a fermentative strain found in the rhizosphere of plants, such as tomato, was the first strain reported to produce metabolites from three known families of *Bacillus* BSFs (surfactin, iturins and fengycin) in anaerobic conditions[61]. Fengycins are peptides composed by 10 α-amino acids, similar to surfactin, in which the decapeptide is linked to a β-hydroxy fatty acid from C_{16} to C_{19} [59]. In anaerobic cultures, total BSF production rate was more than double than in aerobic cultures. The proportion between different BSF was similar in both conditions with surfactin being the most abundant (67 %), followed by iturins (29 %) and fengycin (4%)[61].

1.4.3. *Pseudomonas*

Pseudomonas includes the species *P. aeruginosa,* an opportunistic human pathogen. *P. aeruginosa* infections are often accompanied by formation of biofilms, e.g. in respiratory tract of cystic fibrosis patients[6,62]. As such, it is considered to be a model organism for the study of formation, maintenance and dispersal (also known as detachment) of biofilms[17]. In *P. aeruginosa*, BSF

production is involved in the regulation of biofilm development[63]. Rhamnolipids produced by *P. aeruginosa* and other pseudomonads, are glycolipids composed of a single or dimer of β-hydroxy fatty acid glycosylated to a mono- or di- rhamnose (Figure 1.1). The fatty acid chains can range from C_8 to C_{12} [64]. Rhamnolipids have been found to deeply affect all the three stages of biofilm development. This is mainly due to their involvement in quorum-sensing mechanism, a cell-density-dependent type of cell-to-cell communication[7]. Rhamnolipids are involved in early biofilm microcolony formation[65], while in later stages, they are fundamental in the maintenance of the biofilm architecture[17]. The typical architecture of single-species *P. aeruginosa* biofilms corresponds to several micro-colonies, which in mature biofilms form mushroom-like structures separated by open channels, attached to a surface. Fluids can circulate through biofilm channels, delivering nutrients and TEAs, and removing metabolic products[66]. Rhamnolipids are involved in the maintenance of open channels in mature biofilms, by affecting interactions between cells, or between cells and the interface or solid surface[66] and preventing competitors from adhering to the open areas of the channels[67]. Rhamnolipids are also responsible for the formation of the cap in mushroom-like structures[65,68] and contribute to the dispersion of cells by causing the formation of cavities in the center of the biofilm, from which cells detach[69,70]. Most studies of BSF production on pseudomonads have been conducted in aerobic conditions. However, Yoon *et al.* (2002) reported that *P. aeruginosa* not only forms robust biofilms under anaerobiosis but also prefers anaerobiosis for biofilm development[71]. Even biofilms produced in aerobic conditions have large anoxic zones, because oxygen diffusion is limited to the upper 50-90 μm[72], which points to the possibility of rhamnolipid production under biofilm anaerobic conditions. *In vitro* anaerobic biofilms are thicker and more compact than aerobic counterparts[71], which may indicate a deregulation of the formation of the mushroom-like structures and the maintenance of the fluid channels, which are regulated by the production of rhamnolipids. Nonetheless, well separated micro-colonies have been observed *in vivo*, although the techniques used were not suitable for the observation of mushroom-like structures[62]. Therefore, the production rhamnolipids in anaerobic biofilms is still to be confirmed.

Anaerobic rhamnolipid production has been detected in *P. aeruginosa* PAO1 T, a NRB, related to its swarming ability in high agar (1.5-2.5 %) medium[73]. Swarming is a kind of flagellum-dependent motility on solid surfaces. In this case mutants lacking both flagellum and pili were still able to spread in high agar plates. In *P. aeruginosa,* rhamnolipid is required for swarming, since mutants lacking the *rhlA* gene, required for rhamnolipid production, display a non-swarming phenotype. Swarming activity was restored with insertion of the lacking gene or addition of bio- or synthetic surfactants to the medium. Interestingly, swarming in agar plates was enhanced in anaerobiosis with 8% CO_2[73].

The formation of dense and voluminous foam, intrinsically related to the presence of oxygen, is a problem associated with aerobic fermentation for rhamnolipid production and several attempts of rhamnolipid production in denitrifying conditions have been made in order to circumvent foam production and oxygen limitation. *P. aeruginosa* ATCC 10145 in planktonic state presented an average rhamnolipid production rate of 2 $mg/g_{cell\ protein} \cdot h$ when cultured in denitrifying conditions, which corresponded to approximately 1/3 of the aerobic production. However, the production process could be optimized with higher cell concentrations, since little or no foam was formed and there was no oxygen limitation[74]. The immobilization of *P. aeruginosa* E03-40 in hollow-fiber bioreactors working under denitrifying conditions has also been applied as a strategy to reduce foaming[75]. The BSF was produced for over 1500 h with a specific productivity of 17 $mg/g_{biomass} \cdot h$, similar to that of aerobic conditions. In an initial phase, the bioreactor presented microaerobic conditions and in a later phase, surface aeration was removed so that the conditions were as close to anaerobiosis as possible. However the mass transfer of the BSF through the membrane was affected by concentrations above the BSF CMC due to the formation of micelles and other aggregates[75]. This method coupled with continuous extraction of rhamnolipids may prove to be an interesting alternative for industrial rhamnolipid, and possibly other BSFs, production.

Two BSF-producing hydrocarbonoclastic bacteria isolated from enrichment cultures of contaminated soil and crude oil were identified as *Pseudomonas* sp. BS2201 and *Pseudomonas* sp. BS2203 based on their biochemical characteristics[76]. These are some of the first strains described to be able to produce BSF under both aerobic and anaerobic conditions using crude oil as sole carbon source[76].

Pseudomonas sp. ANBIOSURF-1 was isolated from enrichment cultures of sewage sludge and initially showed potential for MEOR strategies due to its ability to produce BSF in anaerobic environments[77]. It should be noted that in pre-culture anaerobic conditions, sulfate was the only TEA added to the medium, while in anaerobic BSF production nitrate was also added. Further tests revealed that the BSF produced using coconut oil as substrate was a glycolipid, most likely a pure mono rhamnolipid with a CMC of 52 mg/L. Furthermore, the BSF presented better emulsifying ability when tested with chlorinated compounds than with petroleum hydrocarbons (PHs)[77].

The genetic engineering of the facultative anaerobe *Pseudomonas stutzeri* DQ1 by the addition of the rhamnosyltransferase gene *rhlABRI* from the aerobe *P. aeruginosa* SQ6 resulted in a *P. stutzeri*, strain Rhl, able to reduce nitrate and effectively produce rhamnolipids in anaerobic conditions[27]. Through the use of statistical modelling, key nutrients for rhamnolipids production were identified and medium composition was optimized leading to a BSF yield of 3.12 g/L[78]. Like for most facultative anaerobes, growth was faster and biomass and BSF yields were higher under aerobic

conditions. BSF was produced almost in parallel with cell growth during exponential phase (24 h), reaching maximum concentration (3.10 g/L) during stationary phase[78]. Furthermore, thin layer chromatography and Fourier transform infrared spectroscopy confirmed that the rhamnolipid produced by *P. stutzeri* Rhl was similar to that produced by the original carrier of the gene *rhlABRI,* with a CMC of 90 mg/L and highly active within a wide range of temperatures, salinities and pH (Table 1.3, section 1.10)[27].

Two BSF-producing facultative anaerobic *P. aeruginosa* strains, SG and WJ-1, were isolated from oil reservoirs[79]. The BSF produced aerobically by the WJ-1 strain presented a CMC of 14 mg/L[80]. While the rhamnolipid anaerobically produced by the *P. aeruginosa* SG presented a CMC of 80 mg/L and maintained its activity under different pH, temperature and pressure values (Table 1.3, section 1.10)[81]. *P. aeruginosa* SG itself was shown to produce BSF under wide ranges of temperatures, pH and salinities (Table 1.2, section 1.10)[81]. *P. aeruginosa* SG was used to test the effect of increasing the number of *rhlAB* genes, responsible for expression of rhamnosyltransferases, and the replacement of the original promoter for a stronger one, in rhamnolipid production under both aerobic and anaerobic conditions. Two modified strains were tested: *P. aeruginosa* PrhlAB corresponding to the transformed *P. aeruginosa* SG with the recombinant plasmid pBBRPrhlAB, which contains *rhlAB* genes with native promoter, and *P. aeruginosa* PoprAB containing the recombinant plasmid pBBRPoprAB carrying *rhlAB* genes and the strong promoter of *oprL* gene, responsible for the expression of peptidoglycan-associated lipoproteins[82]. BSF production started at early exponential phase, peaking at the end of the stationary phase in all tested strains. Increasing the number of *rhlAB* gene copies resulted in a 1.47-fold increase of BSF produced by strain PrhlAB in comparison to the wild type, strain SG, while the cumulative effect of increasing the number of copies and adding a strong indigenous promotor led to a 3.30-fold increase, by strain PoprAB, under anaerobic conditions[82]. These strategies have proven that overexpression of important genes associated with BSF production is possible even under oxygen-limiting condition. Thus the use of genetically modified anaerobic BSF producing bacteria may lead to improved BSF yields. Potentially making these bacteria more enticing to be used in biotechnological applications.

1.4.4. *Anaerophaga*

Two halo- and thermotolerant strict anaerobes able to produce BSF were isolated from oil contaminated sedimentary tanks in Germany. Both isolates have been initially classified as belonging to the *Bacteroides* genus[83] but later assigned to the new genus *Anaerophaga*[84]. One of the isolates was identified as *Anaerophaga thermohalophila* Fru22 (DSM 12881). The other isolate, identified as Glc12, presents very similar physiological and phenotypic characteristic to strain Fru22. These

two isolates were strictly fermentative, not using oxygen, nitrate, sulfate, thiosulfate, sulfite, sulfur or fumarate as TEAs. Production of BSF in anaerobic cultures was initiated in late exponential phase and continued throughout stationary phase. The produced BSFs were not identified but they did not alter the viscosity of culture media and were able to stabilize emulsions[83]. Fru22 BSF had a molecular weight < 12 kDa, was stable at temperatures up to 70 °C during 20 min, presented optimal activity at pH 2 and was susceptible to proteinase K and trypsin, but not lipase. It was described as an oligopeptide, with attached fatty acids and may contain sugar residues[84]. Both strains gather several favorable characteristics for applications: high temperature and salt tolerance, lack of formation of gases from fermentation, including CO_2, fast growth and production of a thermotolerant BSF[83,84]. However, a setback is that strain Fru22 was not able to grow on solid media[84].

1.4.5. *Bretibacillus*

Bretibacillus sp. BS2202 is a nitrate-reducing hydrocarbonoclastic bacteria isolated together with two *Pseudomonas* strains from oil and oil-contaminated sediments. As the other two isolates, it was able to metabolize PHs and produce BSF both under aerobic and anaerobic conditions. Albeit, the decrease of surface tension in anaerobic conditions was smaller than in aerobiosis[76].

1.4.6. *Clostridium*

One of the first anaerobic BSF producers identified was a *Clostridium pasteurianum* strain. This fermentative bacterium was able to produce a BSF under anaerobiosis using sucrose as a carbon source. The BSF was able to reduce the surface tension of the growth medium to 55 mN/m[85].

1.4.7. *Desulfovibrio*

La Rivière (1955) showed for the first time that a bacterium, the sulfate-reducing bacterium (SRB) *Desulfovibrio desulfuricans* El Agheila Z, could produce BSF under anaerobic conditions. A decrease of surface tension of the medium of 21 mN/m in 7 days was reported. The author hypothesized that *D. desulfuricans* released surfactant to the medium not only through excretion but also by autolysis[37].

1.4.8. *Geobacillus*

Geobacillus pallidus H9 is a halo- and thermotolerant NRB, isolated from a Chinese oil reservoir [86]. It was able to produce BSF under both aerobic and anaerobic conditions, although growth and BSF yields were higher in the former. BSF composition changed in relation to the oxygenation conditions. In BSFs produced under aerobic and anaerobic conditions, the fractions of glycosides

were 50.3 and 53.8% (w/w surfactant), the lipids were 34.5 and 31.2% and the peptides represented 15.2 and 26.0%, respectively. This corresponded to slightly different CMC values for the BSF produced in aerobiosis (16 mg/L) and anaerobiosis (22 mg/L) and by a stronger reduction of medium surface tension in aerobic cultures (Table 1.1, section 1.10). BSF yield, cell surface lipophilicity and emulsification capacity were negatively affected by anaerobiosis. The maximum BSF yields were of 9.80 g/L and 2.16 g/L, cell surface lipophilicity were 88.4% and 64% and emulsification indexes at 24 h were 90-95% and 10-35% for the BSF produced in the presence or in the absence of oxygen, respectively[86].

1.4.9. *Rhodococcus*

Species of *Rhodococcus* genus are known aerobic producers of intracellular BSF. *Rhodococcus ruber* Z25, a NRB isolated from an oil well in China was able to grow and produce BSF in anaerobic conditions, albeit presenting much lower biomass and BSF concentrations (0.11 g/L and 0.53 g/L, respectively) than those achieved in aerobic cultures (1.46 g/L and 12.95 g/L, respectively)[87].

1.4.10. *Thermoanaerobacter*

Yen *et al.* (1991) demonstrated the production of an extracellular glycopeptide BSF by *Thermoanaerobacter pseudethanolicus* 39E, formerly *Clostridium thermohydrosulfuricum* 39E, under anaerobiosis. Isolated from hot springs, this extremophile is a strict anaerobe with very promising characteristics for use in biotechnological applications, since it is thermophilic, halotolerant and spore forming[88]. This is one of the few SRB known to produce BSF in oxygen-limiting conditions.

A mixed culture, predominantly composed by a *Thermoanaerobacter* strain, isolated from heavy oil samples has been shown to produce BSF at reservoir conditions (70 °C, 15 g/L NaCl) and using molasses as substrate. Additionally this culture was able to grow under pressures of 0.8 to 14.2 MPa and production of methane was observed[89].

1.5. Effect of oxygen depletion on BSF production pathways

Very little is known about the regulation of BSF production under microaerobic or anaerobic conditions. It is assumed that metabolic pathways are identical to those operating in aerobic conditions[49] and comprehensive descriptions of the genetics and biochemistry of bacterial BSF production can be found in specific reviews[64,90–92].

Two of the most common BSFs produced under oxygen-limiting conditions are surfactin and rhamnolipids, by *Bacillus* and *Pseudomonas* species, respectively. In general, the biosynthesis of surfactin by *Bacillus* is thought to be non-ribossomal, depending instead on the activity of peptide-synthetases. These multienzyme complexes catalyze the synthesis of peptides using directly precursor amino acids without any need for ribosomal protein synthesis. Biosynthesis of surfactin ends when the molecule becomes cyclic or is released by the complex. Operon *srf* contains the genes responsible for the regulation of surfactin synthesis and encodes for some of the enzymes subunits[48,91]. Rhamnolipids produced by *Pseudomonas* species are mainly regulated by the *rhl* quorum-sensing system. The biosynthesis of rhamnolipids is the result of the junction of two pathways, the biosynthesis of dTDP-L-rhamnose and the biosynthesis of the fatty acid moiety, by two consecutive rhamnosyl-transfer reactions. Each reaction is catalyzed by a specific rhamnosyltransferase. The first is encoded by the genes *rhl A* and *B* and the second by the gene *rhlC*[91,92].

Some studies addressed specifically the influence of oxygen depletion on BSF production. In the previously mentioned *P. stutzeri* Rhl, the ability to produce BSF was added to a facultative anaerobic non-BSF producing *P. stutzeri* strain[27]. This was interpreted as evidence of rhamnolipid production in pseudomonads not being directly dependent on oxygen, even if oxygen availability is likely to influence other metabolic pathways, namely those related with organic carbon consumption[74]. Furthermore, it should be noted that the original strains, *P. aeruginosa* SQ6 and *P. stutzeri* DQ1, are close relatives and may share regulatory mechanisms[27].

Even if BSF production is not directly dependent on oxygen, there is evidence that the absence of oxygen can indirectly affect the metabolic pathways involved in the production of BSF. In several cases BSFs produced under aerobic and anaerobic conditions differ in the relative proportion of different components[28,86]. It has been hypothesized that under anaerobic conditions, substrate preferences change due to the redox potential of the biochemical reactions. This, coupled with the usage of different electron acceptors, may lead to changes in the metabolic pathways involved in the production of BSF.

In a study conducted during a spaceflight mission, a transformed *P. aeruginosa* PAO1 resistant to gentamicin adopted anaerobic growth[93]. The culture was kept inside the spacecraft, under aerobic conditions, but exposed to microgravity and low fluid shear, resulting in a less efficient oxygenation of the liquid culture medium than in the control, incubated on Earth. Most genes upregulated in relation to the control were related with anaerobic growth, especially denitrification. Additionally, gene *rhlA*, involved in the synthesis of rhamnolipids, was among the most upregulated genes. However, gene *rhlI*, responsible for the N-butanoyl-L-homoserine lactone synthase and also

involved in rhamnolipid production, was down regulated[93]. Furthermore, biofilm formation by *P. aeruginosa* was observed during the spaceflight, although the presence of rhamnolipids was not directly confirmed[94]. The combination of transcriptomic and metabolomics approaches could shed some light on the regulation of BSF synthesis in sub-oxic conditions.

Generally, the isolates listed on Table 1.1 (section 1.10) present higher BSF yields under aerobiosis than in oxygen-limiting conditions. Primary metabolic pathways that are not dependent on O_2 produce less energy than their aerobic counterparts[95]. Since BSFs are usually secondary metabolites[55], it is possible that their production is reduced under oxygen-limiting conditions considering that the available energy is preferentially used in vital metabolic pathways instead of BSF production. On the other hand, less energy results in lower cell growth which implicates less BSF production when BSF production is growth-dependent (further discussed later in this review). In cases where BSF yields are higher under oxygen-limiting conditions[54,60,61,96] it is possible that non-vital pathways that were being preferentially used in aerobic conditions are now less used and the energy redirected towards BSF production.

Because changes in BSFs composition affect their properties, such as the CMC and emulsification index[86], it is important to understand, from the molecular perspective, how and why these shifts occur, in order to design efficient cultivation conditions to achieve specific production outcomes. Unfortunately, this information is still very scarce in scientific literature.

1.6. Growth conditions and optimization of media for BSF production under oxygen-limiting conditions

Bacterial growth is affected by several factors, among which the composition of the culture medium is extremely significant. The optimal growth conditions for BSF production should achieve the highest yields and the best product quality. A scheme summarizing the main effects each factor can have on BSF production in oxygen-limiting conditions is presented in Figure 1.2.

1.6.1. Aerobic, microaerobic or anaerobic conditions

Some bacteria produce higher amounts of BSF under low oxygen availability. Strict anaerobiosis can be achieved in several ways, but most often by addition of a chemical reductant to the culture medium, most frequently sodium sulfide (Na_2S), cysteine-HCl or ascorbate[97,98]. The choice depends on several parameters such as the degree of reduction of medium intended, the possibility of being used as a carbon source, the formation of precipitates with other compounds or bacterial toxicity.

A concentration of 2.0 g/L of Na_2S was reported as the most adequate for anaerobic production of BSF by *B. licheniformis* VKM B-511[54]. On the other hand, the use of a solution of cysteine-HCl and $Na_2S{\cdot}9H_20$ as chemical reducing agents showed to negatively affect growth and BSF production in *B. mojavensis* JF-2, because sulfide inhibits the activity of key enzymes in some NRB[41]. Production of lichenysin B by *B. mojavensis* JF-2 was the highest with 30% dissolved oxygen in media with 2% (w/v) NaCl, although biomass production is higher with 85% oxygen saturation[96]. Other studies showed the production of acceptable levels of BSF in *B. mojavensis* JF-2 under anaerobiosis[41,99]. Hence, it was proposed that BSF production by *B. mojavensis* JF-2 may be more dependent on growth rate than on O_2 availability, although the growth rate is influenced by dissolved oxygen and NaCl concentrations. As such, slower growth would lead to a longer exponential phase, when the BSF is produced and released, and a later stationary phase, when uptake of BSF occurs[96]. Other bacterial species have maximum BSF production yields under anaerobic and microaerobic conditions. That is the case of *B. licheniformis* VKM B-511[54] and *B. subtilis* C9[60]. *B. amyloliquefaciens* S499 also presents a higher BSF production rate in anaerobiosis than aerobiosis[61].

A bioreactor study with *B. subtilis* ATCC 21332 demonstrated that surfactin production is affected by aeration and agitation. Optimized surfactin production conditions, 1.5 vvm and 300 rpm,

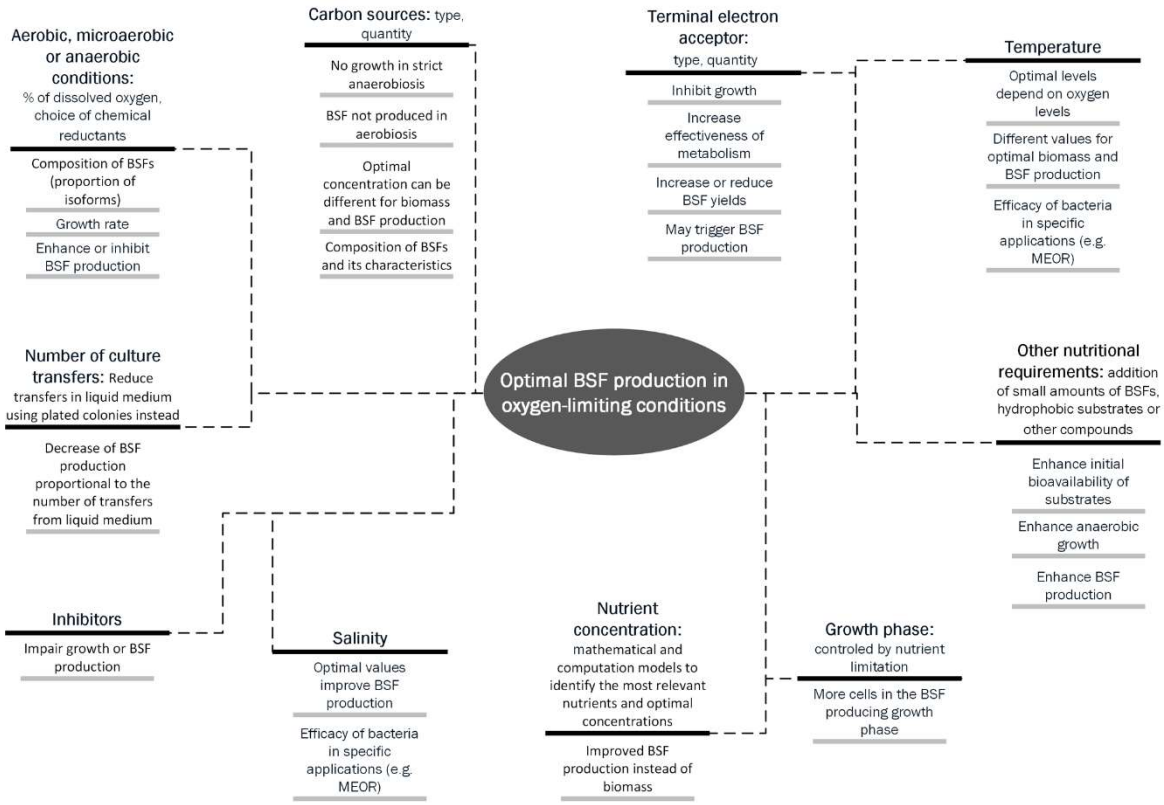

Figure 1.2 – Main parameters that can affect BSF production in oxygen-limiting conditions and their possible effects.

resulted in an almost complete exhaustion of O_2 during exponential growth, with surfactin concentration reaching 6.45 g/L and surfactin production yields of 161 $mg/g_{glucose}$[100].

Oxygenation also affects the composition of the BSF produced. A study addressed the regulating effect of oxygen in the production of mycosubtilin, and to a smaller extent surfactin in *B. subtilis* ATCC6633 and *B. subtilis* BBG100[56]. An increase of 25-fold in mycosubtilin production was observed in *B. subtilis* ATCC6633 cultures with the lowest oxygen rate transfer tested, 7 mmol $O_2/L \cdot h$, in comparison to the highest, 20 mmol $O_2/L \cdot h$. This resulted in an increase in mycosubtilin concentration of 2.8 mg/L to 45.4 mg/L and mycosubtilin specific productivity from 0.001 $mg/g_{biomass} \cdot h$ to 0.025 $mg/g_{biomass} \cdot h$, from the highest tested oxygen rate transfer to the lowest. In *B. subtilis* BBG100 cultures both mycosubtilin concentration (67.9-82.2 mg/L) and specific productivity (0.033-0.037 $mg/g_{biomass} \cdot h$), remained similar independently of the oxygen rate transfer. Mycosubtilin and surfactin metabolisms share an identical cofactor, which can limit surfactin production when mycosubtilin is over produced. The native promoter of mycosubtilin synthetase operon was shown to be at least in part, responsible for the oxygen regulation of mycosubtilin production. *B. subtilis* ATCC6633, which contains the native promoter, is affected by oxygen rate transfer and *B. subtilis* BBG100, which contains an exogenous promoter, is barely affected. Additionally, differences in the production of BSF isoforms were reported, with an increase in the percentage of mycosubtilin with C_{17} fatty acid chains and a decrease in the C_{16} isoform with increasingly microaerobic conditions. This indicates that oxygen may also be involved in regulating the production of lipopeptides[56]. Likewise, surfactin produced under aerobic and anaerobic conditions can have different percentage of isoforms. HPLC spectra of surfactin produced by *B. subtilis* ATCC 21332 show the same five isoforms present for both BSFs, but the mass concentration of each isoform varies. When the recovered surfactins were tested, induction of gas hydrate formation was significantly more effective for surfactins produced under anaerobic conditions[28]. Therefore, differences in the proportion of isoforms present affect BSF activity.

In *G. pallidus* H9, substrate uptake varies between aerobic and anaerobic conditions. This, is believed to cause differences in the constitution of the BSF produced, which resulted in different values of CMC, cell surface lipophilicity and emulsification[86].

The optimal conditions for growth and spreading of *P. aeruginosa* PAO1 T, in which swarming is rhamnolipid-dependent, are anaerobic conditions supplemented with 8 % CO_2[73].

It should be noted that some bacteria that produce BSF under aerobiosis may completely fail to do it under oxygen deprivation. Two *P. aeruginosa* strains that produced BSFs with good emulsifying activity in aerobic conditions, were able to grow in the exact same medium under anaerobiosis but neither a decrease of surface tension nor emulsification were detected[26]. The same

was observed in another study with *P. aeruginosa* CVCM 411[101]. In other cases, the time of production under anaerobic conditions could significantly increase. Additionally, BSF and biomass yields are also usually lower under anaerobic conditions. As an example, production of BSF both in PPGA medium and MSM with crude oil by three strains, two *Pseudomonas* sp. and one *Brevibacillus* sp., took 10 days in aerobic conditions and 50 days in anaerobiosis[76]. Moreover even with the extra reactional time, usually surface tensions of anaerobic media at the end of fermentation are never as low as values obtained under aerobic conditions[76], which could be a consequence of reduced BSF production.

In general, oxygen availability can affect BSF production, rate and yield as well as BSF composition and isoform percentage, and consequently, overall BSF properties. Therefore, oxygenation is a critical parameter in BSF production with considerable impact in the efficiency of the process and on quantity and quality of BSF produced.

1.6.2. Terminal electron acceptors

The most common TEAs used by bacteria under anaerobic conditions are sulfate (SO_4^{2-}) and nitrate (NO_3^-). However, some other less common TEAs, such as manganese (Mn^{4+}), nitrite (NO_2^-), sulfur (S^0), sulfite (SO_3^{2-}), carbonate (CO_3^{2-}), iron (Fe^{3+}), carbon dioxide (CO_2) or fumarate, may additionally be used[35]. All BSF producers capable of operating anaerobic respiration described in this review are either SRB, NRB or use organic compounds as TEAs (Table 1.2, section 1.10).

P. aeruginosa growing under anaerobic conditions preferably uses nitrate as TEA[102], although high concentrations of nitrate may inhibit growth[103,104]. It can also use nitrite or arginine as alternative acceptors[102]. Surfactin batch production by *B. subtilis* ATCC 21332 also relies on nitrate reduction[55]. Under anaerobiosis nitrate was used as the preferred TEA and once depleted, nitrite was used. This transition actually corresponded to an enhancement in BSF production. When nitrate was not limiting, nitrite is not used, resulting in lower BSF yields[55]. In this case, it appears that the increase in BSF production was not directly triggered by oxygen depletion *per se* but rather by the use of nitrate, which the authors believe to be involved in signaling the start of the secondary metabolism related to the production of BSF by the bacteria[55]. In an anaerobic study aiming to assess the impact of addition of nitrate in the microbial community, oil souring and potential for MEOR application, BSF production was detected. This consisted in water flooding experiments in sand packed columns inoculated with a microbial consortium isolated from a Brazilian offshore oil reservoir[105]. The produced BSF, which was detected by a decrease in interfacial tension, considered to be partly responsible for the increase of 4.3% (v/v) of the initial oil recovered when compared to the culture without added nitrate[105].

1.6.3. Carbon sources

Not all carbon sources used by a facultative anaerobe in aerobic conditions can be used during anaerobic growth. This happens because the metabolic pathways of some substrates require oxygen to be functional. As an example, the metabolization of hexadecane by *P. aeruginosa* ATCC 10145 for the production of rhamnolipids, was only possible in the presence of oxygen[74,103]. When alternative carbon sources were tested (palmitic acid, stearic acid, oleic acid, linoleic acid, glycerol, vegetable oil and glucose) anaerobic production of rhamnolipids was only observed in the cultures with palmitic or stearic acids. Palmitic acid rendered the best production which, albeit, in aerobic conditions, it corresponded at the best, to 82% of the productivity achieved with hexadecane[74]. Therefore, the selection of the carbon source for anaerobic cultures should take into account not only the capacity of bacteria to metabolize it in absence of O_2 but also the BSF production efficiency of the culture.

While in most anaerobic studies either sugars or PHs are used as substrates, other lower cost substrates, such as vegetable oils, can be used. Anaerobic rhamnolipid production was documented in *Pseudomonas* sp. ANBIOSURF-1 growing on coconut oil[77]. Glycerol, a byproduct of biodiesel and soap production, was also identified as the carbon source that achieved lower medium surface tension values in anaerobic *P. aeruginosa* SG cultures. Other low cost substrates that led to a less effective production of anaerobic BSF by *P. aeruginosa* SG were sunflower oil and soybean oil[81,104].

Substrate concentration can also influence biomass and metabolite production, including BSF. Tests performed in a mixed culture composed mainly of *Thermoanaerobacter* sp. revealed that maximum biomass and CO_2 yields were obtained at concentrations of glucose < 1.68 g/L. However, BSF production was only observed at initial substrate concentration above 5.8 g/L[89]. In a study addressing the production of rhamnolipids by *P. aeruginosa* SG the best production yield was obtained at glycerol concentration of 72 g/L, and production yield was positively correlated with the concentration of carbon source within the range of 12 g/L up to 72 g/L. These studies indicate that high yields of BSFs require considerable concentrations of carbon sources[104].

Substrate type affects the properties of the produced BSF. Studies on the effect of carbon source in *B. subtilis* C9 showed that different carbon sources affected BSF production, surface tension of the medium and emulsification activity of the broth. Out of several media with different carbon sources, emulsification of crude oil was only observed in glucose broth, which was also the medium showing lowest surface tension and highest BSF yield and production rate. Authors noted a direct correlation between BSF production, bacterial growth and glucose uptake. However, concentrations of glucose higher than 40 g/L had little effect on BSF production[60].

PHs have also been used as substrates by BSF producing bacteria. Five *B. subtilis* strains were able to grow and produce BSF in anaerobic conditions using a mixture of long-chained alkanes (C_{20}-C_{30}) as sole carbon source[26]. Gas chromatography analysis showed that most of the tested strains were able to degrade *n*-alkanes with C_{27} or higher and some were also able to degrade *n*-alkanes between C_{18} and C_{20}. Furthermore, the addition of *n*-hexadecane to a rich medium did not affect anaerobic BSF production[26]. *G. pallidus* H9 was able to grow with crude oil as carbon source and produce BSF both under aerobic and anaerobic conditions[86]. In aerobic cultures, medium- and long-chained hydrocarbons (C_{23}-C_{43}) were used preferentially, with hydrocarbons C_{41}-C_{43}, which were initially present in smaller concentrations, being completely metabolized. In anaerobic cultures, an overall preference for small- (C_8-C_{11}) and long-chained hydrocarbons (C_{32}-C_{43}) was observed and the hydrocarbon fraction C_{39}-C_{43} was totally degraded. Consequently, differences in the BSF composition were observed. BSF with different CMC, 16 mg/L and 22 mg/L, and emulsification indexes of 90-95% and 10-35%, were obtained in aerobic and anaerobic conditions, respectively. The anaerobically-produced BSF presented higher glycosidic and peptidic fractions and lower lipidic fractions than the BSF produced in aerobic conditions[86].

1.6.4. Other nutritional requirements

In addition to bulk carbon sources, some bacteria have other nutritional requirements for growth or production of BSF in anaerobiosis or microaerobiosis (Table 1.2, section 1.10). *B. mojavensis* JF-2 requires yeast extract to grow anaerobically in mineral modified medium E[41]. Additionally it also requires deoxyribonucleosides or DNA to grow under strict anaerobic conditions. No such requirements are observed in aerobic growth of this strain. This is thought to be due to the lack of ribonucleotide reductases able to operate in anaerobiosis, which are needed to convert ribonucleotides into deoxyribonucleotides [106]. Addition of tryptone, proteose peptone or neopeptone has also been reported to be critical for anaerobic growth and BSF production in several bacteria[89].

Small amounts of BSF may be initially added to the culture medium to enhance solubility and bioavailability of the substrate and shorten the adaptation time of bacteria. However, some bacteria may also metabolize the added BSF which will actually provide a supplementary carbon source[74]. In the case of highly soluble carbon sources, the addition of small amounts of hydrophobic substrates, such as hexadecane, can serve as an additional stimulation and improve BSF production[83].

Ions, particularly PO_4^{3-}, SO_4^{2-}, and Mg^{2+}, have been found to positively affect anaerobic rhamnolipid production by *P. aeruginosa* SG. Phosphate concentration in particular was found to be linked to BSF production yield[104]. This anion is required for the formation of thymidine diphosphate

(dTDP), which is essential to one of the rhamnolipids' precursors, dTDP-L- rhamnose[92]. SO_4^{2-}, and Mg^{2+} are thought to be cofactors in enzymes involved in the rhamnolipid production pathways[104].

1.6.5. Nutrient concentration

The proportion in which particular supplements are provided also affect anaerobic or microaerobic BSF production. C/N ratios affect bacterial growth and BSF production and can be as important as the nature of the carbon or nitrogen sources. In a comprehensive study of the optimal BSF production conditions of *B. licheniformis* VKM B-511 optimal production yields were achieved with a 1:24 ratio[54]. In a similar study, the optimal C/N ratio for rhamnolipid production by *P. aeruginosa* SG was estimated as 1:16[104].

A recent approach to media optimization used mathematical and computational models to provide predictions regarding cell growth and metabolites yields. This approach has been used for optimization of medium for anaerobic production of rhamnolipids by *P. stutzeri* Rhl[78]. In this case the most significant variables in the media composition were identified as glycerol, yeast extract and KH_2PO_4. Further analysis was performed to determine optimal concentrations. The fermentations used for the validation of the model obtained an average BSF yield (3.12 g/L) very close to the maximum predicted (3.26 g/L) and the model was, therefore, considered to be accurate and reliable. The optimized medium almost doubled the BSF yield when compared to the original medium (1.68 g/L)[78]. In a similar study with *P. aeruginosa* SG, by the same group, glycerol, $NaNO_3$ and phosphate were identified as the significant variables in anaerobic production of rhamnolipid. An increase of 1.71-fold was observed when optimal medium was used instead of the initial one. Also in this case the estimated BSF yield (632.3 mg/L) obtained using optimal medium was close to the experimental one (650.1 mg/L)[104]. Therefore the application of mathematical and computational models to the optimization of BSF production in anaerobiosis can be a powerful tool, especially advantageous in reducing costs in trial and error experiments in medium formulation. However it should be employed with a critical frame of mind and model validation should always be performed.

1.6.6. Inhibitors

Inhibition of anaerobic BSF production by particular compounds has been reported (Table 1.2, section 1.10). Crude oil was found to decrease biomass, BSF and polymer production on *B. licheniformis* BNP29[50]. This was a surprising observation since the strain was isolated from an oil reservoir and the presence of hydrocarbons or compounds with low bioavailability is usually related with increased BSF production.

In a study aiming to optimize culture media for anaerobic BSF production, organic sources of nitrogen such as yeast extract and peptone, had an inhibitory effect in anaerobic rhamnolipid production by *P. aeruginosa* SG[104]. It is thought that since these sources can also be used as carbon sources they may not be as conductive to BSF production as nutrient-limiting conditions, which are easier to achieve using nitrate as nitrogen source[104]. In the same study hydrophobic substrates were found to have a negative effect in the production of BSFs[104].

Sulfide is also known to directly, yet reversibly, inhibit BSF production[79,107]. As previously mentioned, sodium sulfide can be added as a chemical reductant to anaerobic media, however another source of sulfide that can affect bacteria in natural environments is hydrogen sulfide produced by SRB. *P. stutzeri* Rhl is tolerant to initial sulfide concentrations up to 33.3 mg/L, at higher concentrations sulfide removal decreases drastically and almost no BSF is produced[107]. *P. aeruginosa* strains SG and WJ-1 are even less tolerant, with growth and BSF production being inhibited at sulfide concentrations above 10 mg/L, and unable to remove sulfide from medium[79]. Increasing the volume of inoculum of BSF-producing bacteria attenuated the inhibitory effect of sulfide[79]. Such a decrease in BSF production is thought to be the result of cell growth inhibition[79]. Sulfide inhibition of anaerobic BSF production can be of particular importance to the success of MEOR strategies, as is discussed in a latter section.

1.6.7. Salinity

BSF production in marine bacteria is widely documented in scientific literature and anaerobic halotolerant BSF-producing bacteria are of particular interest in MEOR strategies, since oil reservoirs often present high salinity and low oxygen concentrations. Salinity concentrations tolerated by the different known BSF producing-bacteria under oxygen-limiting conditions and optimal values for BSF production are presented in Table 1.2 (section 1.10). The table shows that known microaerobic and anaerobic BSF producers' salinity requirements range from zero to 150 g/L NaCl. In addition, the majority of the most halotolerant bacteria listed were previously isolated from crude oil[26,50]. Only few studies have assessed the isolates optimal salinity concentration for BSF production. In all cases listed optimal salinity concentration for BSF production coincides with the optimal salinity for growth[39,54]. Since salinity is known to affect BSF yields[39,54,99], it is therefore an important parameter for optimization of BSF production.

1.6.8. Temperature

Optimal growth temperature of BSF-producing bacteria (Table 1.2, section 1.10) can differ depending on oxygenation. In *G. pallidus* H9 growth occurred between 40 °C and 80 °C, but optimal

growth temperature was 65 °C under aerobic conditions and 70 °C under anaerobiosis[86]. However, little is known on the direct effect of temperature in anaerobic BSF production[26,86,89]. Yakimov *et al.* (1995) observed that *B. licheniformis* BAS50 lichenysin A and biomass yields in anaerobic cultures were affected by temperature. The highest production was achieved at 40-45 °C[39]. Likewise, *B. licheniformis* VKM B-511 was able to grow in a range of temperatures from 20 °C to 40 °C. BSF yield was the highest at 30 °C even though maximum growth rate was achieved at 40 °C[54]. This demonstrates that optimal growth temperature and optimal temperature for anaerobic BSF production are not always the same.

Determination of optimal anaerobic growth and BSF production temperatures are important in industrial production of BSFs as well as in *in situ* bioremediation and MEOR strategies. In the first case to improve production. In the latter, because some of the targeted environments for application of MEOR or bioremediation strategies can present extreme temperatures, such as high-temperature oil reservoirs[89] or sediments in cold regions.

1.6.9. Growth phase

In most reports of BSF-producing bacteria under oxygen-limiting conditions, BSF production is dependent on growth phase. In all strains in which BSF production under oxygen-limiting conditions was studied alongside the growth curve, BSF production started during the exponential phase. Probably, indicating that the produced BSFs are secondary metabolites. In some cases BSF production peaked during the exponential phase and its presence is reduced during stationary phase[41,50,60,86] while in others, BSF production reached its maximum during stationary phase[27,55,74,83]. Production of rhamnolipids by *P. aeruginosa* happens mainly in stationary phase and is dependent on cell density. A way of controlling and limiting *P. aeruginosa* growth, and subsequently the BSF production, is to impose nutrient limitation. In aerobic conditions, nitrogen is commonly the chosen limiting nutrient. However, under denitrifying conditions nitrogen is required as TEA and therefore cannot be in limited concentrations in the medium. A study designed to identify an alternative limiting nutrient to be used in BSF production by *P. aeruginosa* ATCC 10145 under anaerobic conditions revealed that Ca and Fe had no effect, Mg had modest effects and S limitation was comparable to N limitation[74]. In an anaerobic batch culture containing nitrate it was observed that *P. aeruginosa* SG started immediately consuming nitrate which led to a quick increase of biomass and the start of rhamnolipid production. However, once nitrate reached limiting concentrations and was eventually depleted the formation rate of the BSF increased[81,104]. BSF production by three strains of *Pseudomonas* was enhanced when grown in phosphate-limiting media with protease peptone and glucose, in comparison to growth in a minimal salts medium with crude oil[76]. Limiting P availability by adding phosphorous-free medium to an anaerobic bioreactor with *P.*

aeruginosa, substantially increased rhamnolipid production[75]. This strategy has also been successfully used in anaerobic surfactin production by *B. subtilis* ATCC 21332. BSF yield in nitrate-limited medium under anaerobic conditions was more than 3 fold higher than in N-limited aerobic conditions[55]. Therefore, nutrient limitation can be an important tool for controlling bacterial growth and increase BSF production in oxygen-limiting industrial conditions.

1.6.10. Number of culture transfers

Some strains seem to have a limit to the number of possible sub-cultivations. Javaheri *et al.* (1985) tested the capacity of *B. mojavensis* JF-2 to produce BSF after several generations, under aerobic and anaerobic conditions. They showed that for both conditions there was an increase of minimum surface tension with increasing numbers of transfers between liquid media[41]. A latter study demonstrated the importance of using fresh inocula with as few serial transfers in liquid medium as possible. With a fresh inoculum of *B. mojavensis* JF-2, up to 24% of residual oil was recovered from a core displacement experience while an inoculum transferred 15 times was only able to recover less than 3%[99]. This loss of productive capacity was accompanied by other phenotypic changes. Normal producers presented volcano-shaped colonies in solid medium and low-activity producers formed round colonies[41]. Plasmids were not detected in normal producers and therefore, the loss of BSF productive capacity could not be attributed to a loss of plasmids[41]. The use of plated colonies as inoculant, in order to reduce the number of liquid media transfers may minimize this problem. Some BSF producers are stimulated by the presence of hydrophobic compounds in the medium[26,83] and therefore, it is possible that the loss of productive activity is related with the release of selective pressure along transfers.

1.7. Applications of BSFs

There is an ever increasing number of applications for BSFs related with industry, health and environment. Given the scope of this review only applications likely to occur under anaerobic or low oxygen conditions will be addressed.

1.7.1. Bioremediation

Bioremediation refers to the removal of a contaminant from a contaminated environment through the mobilization of living organisms and acceleration of the natural biodegradation process. These strategies can be divided in two major groups: bioaugmentation, in which specialized organisms are added to the environment to metabolize the contaminants, and biostimulation, in which nutrients are added to enhance the degradation of the contaminant by endogenous bacteria[108].

The addition or production of BSFs often bring benefits to bioremediation strategies, especially when hydrophobic pollutants are involved, but that is not always the case[23]. BSF addition in some cases may delay the bioremediation process because it is used as a carbon source, thus competing with the pollutant, or for being toxic to the degrading bacteria[23]. For optimal results, BSF-producers for bioaugmentation strategies should be chosen not only in function of their ability to grow under the prevailing environmental conditions, but also considering the properties of the BSF and the effects on autochthonous bacterial community.

Anaerobic sludge reactors used in domestic and industrial wastewater treatment are often spiked with surfactants to enhance the biodegradation of certain compounds[109,110]. However due to environmental concerns, several studies have attempted replacing chemical surfactants with BSFs[111–113]. Similarly, studies of *in situ* bioremediation in river sediments of several xenobiotics, such as decabromodiphenyl ether[114], tetrabromobisphenol-A[115] and tetrachlorobisphenol-A[116], were conducted under anaerobic conditions and achieved promising results with the addition of BSFs, namely surfactin and rhamnolipids. In both strategies, it could be interesting to add directly BSF-producing bacteria which in some cases may also be able to degrade the pollutants and would reduce the process time and economic resources by elimination of extra steps in BSF production and extraction.

Removal of heavy metals from contaminated soil is another possible application of anaerobic BSF-producing bacteria. A previous review discussed the use of BSFs in such technologies, noting that their use has advantages over the use of chemical surfactants as well as the existence of several studies with success[10].

Bioremediation of PHs from natural environments represents one of the most promising applications of anaerobically-produced BSFs. It is often considered that hydrocarbonoclastic bacteria involved in these strategies benefit from the presence of surfactants that enhance the hydrocarbons bioavailability[117]. Due to biodegradability and toxicity issues, BSFs are often preferred to chemical dispersants[23,24]. As such, ideally the hydrocarbonoclastic bacteria are BSF-producers or alternatively, in strategies of bioaugmentation, BSF-producers are added together with the hydrocarbon degraders. While less frequent than the latter, some of most promising BSF-producing hydrocarbonoclastic bacteria to be used under anaerobic conditions include *Pseudomonas* sp. BS2201, *Pseudomonas* sp. BS2203 and *Bretibacillus* sp. BS2202[76], *G. pallidus* H9[86], *Rhodococcus ruber* Z25[87] and several *B. subtilis*[26]. While these bioremediation strategies are usually applied under aerobic conditions, in some cases they are required in anaerobic environments, such as aquatic environments or anaerobic sediment layers. In a study of *in situ* bioremediation of an aquifer contaminated with diesel fuel under denitrifying conditions, the addition of rhamnolipids at concentrations above their CMC, delayed

hydrocarbon degradation because the BSF was used as carbon source by indigenous bacteria[23]. The addition of rhamnolipids also led to an increase in microbial biomass that could potentially clog the pores in soil and hinder the bioremediation strategy. A synthetic surfactant, Triton X-100, was tested as well but also performed poorly by being toxic to biodegrading bacteria. *In situ* anaerobic production of the BSF was proposed as an alternative to overcome the competition with diesel fuel as a carbon source[23].

When the compatibility of lichenysin B produced *ex situ* by *B. mojavensis* JF-2 for anaerobic hydrocarbon bioremediation, was tested under methanogenic, nitrate- and sulfate-reducing conditions[118], overall biodegradation of toluene was not influenced by the BSF, either added or locally produced. However under specific conditions toluene biodegradation was affected. Under methanogenic conditions, toluene biodegradation was inhibited by BSF above CMC levels, while under sulfate-reducing conditions, degradation was stimulated with concentrations of BSF above the CMC and inhibited at concentrations below 1/4 CMC. Hexadecane degradation was observed in all the anaerobic conditions in the presence of BSF at levels above the CMC. However, no difference was noted regarding degradation levels between addition of pre-purified BSF and *in situ* production. No clear trend was observed on the degradation of naphthalene[118,119]. These results alert to the fact that a seemingly promising bacteria for bioremediation may only perform as wished under very specific conditions and for particular pollutants. This may help justify why, while there are several works concerning application of *ex situ*-produced BSFs and some laboratory scale studies about *in situ* BSF production, there is a lack of studies regarding the production of BSF in *in situ* during bioremediation strategies in oxygen-limited environments.

1.7.2. Microbial enhanced oil recovery

Microbial enhanced oil recovery (MEOR) involves biostimulation or bioaugmentation strategies to promote favorable metabolism in bacteria that result in higher percentages of oil recovered from an oil well. Metabolic activities of interest include degradation of paraffin, inhibition of SRBs or production of BSFs, emulsifiers or polymers, among others[120].

The use of surfactants in enhanced oil recovery (EOR) strategies is of particular interest due to their ability to lower interfacial surface tension between the hydrocarbon and the aqueous phases and rock present in oil wells. In normal conditions, the surface tension between these phases is high enough to trap hydrocarbons in the more porous strata, e.g. those including limestone, adjacent to oil wells. Addition of surfactants will therefore lower the surface tension, attenuating capillary forces, and allow for the displacement of oil from the pores by water ultimately resulting in increased oil production rates from oil wells[120]. However, for these approaches to work, surfactant or BSF

concentration inside the reservoir must be well above the CMC levels[51,121]. In cases where surfactants are used considerable amounts are required, which need to be constantly replenished, increasing production costs and eventually rendering the process as economically unviable[51]. BSF are promising alternatives since some of these compounds present higher activity than synthetic surfactants, and only very low concentrations are needed for the process. BSF produced by bacteria generally present very low CMCs, especially when compared with chemical surfactants (2100 mg/L, SDS), including surfactants used in EOR strategies, such as Enordet and Petrostep (both 1 g/L)[53]. However, production of BSF *ex situ,* and later transportation and deployment into the oil fields can still significantly increase oil production costs[122]. A more economical alternative is the production of BSF *in situ* which may eliminate the need of replenishment[29]. MEOR strategies use much less energy than EOR, and are independent on the price of the crude oil[120]. One of the limitations of BSF-based MEOR is that added or locally-produced BSFs may be used as substrate by other bacteria[123] thus decreasing the efficiency of the process and the concentration of BSF may be a key factor in the outcome of the MEOR strategy. The search for new BSF producers is often connected to the development of new and improved MEOR techniques. However, as previously mentioned, most BSF producers have only been tested under aerobic conditions. As such, these producers may not be suitable for *in situ* application in the microaerobic and/or anaerobic environments present in most oil reservoirs[29]. Anaerobic BSF producers can be seen as a viable solution of this problem[27,29]. In addition to the advantages mentioned above, *in situ* strategies using BSF producing anaerobes are easier to implement than other MEOR strategies and do not require modification to the pipeline and equipment. Furthermore, depending on the lithography, bacteria can be spread by underground fluids and positively affect the oil recovery of a wider area[27]. Since most oil reservoirs present high salinity levels[124] and temperature[125,126], anaerobes should also be halo- and thermotolerant. In some instances barotolerance may also be advantageous.

Most known BSF-producers under oxygen-limiting conditions have been isolated from oil wells and/or aimed at application in MEOR strategies[26,41,50,81,89]. Many of these isolates use the PHs as carbon sources[26,86,87]. An advantage of using hydrocarbon-degrading BSF-producing bacteria in MEOR strategies is that no external carbon source is needed. In water flooding experiments in sand packed columns, strategies involving production of BSF by *R. ruber* Z25 *ex situ* were more effective than *in situ* strategies, with initial oil recoveries of 17-28% and 9%, respectively. This is thought to be due to low availability of TEAs[87]. In 100-day crude oil degradation assays with *G. pallidus* H9, decreases in the aromatic, asphaltene and non-hydrocarbon fractions were observed in both aerobic and anaerobic conditions. Because the hydrocarbon fractions taken up by bacteria were not the same in both conditions, BSF composition was also affected, as mentioned previously. Furthermore, cell surface lipophilicity was affected by BSF production, which impacts interactions between cells and

oil. In aerobic conditions, higher cell surface lipophilicity was observed when compared to anaerobic conditions, indicating an increased bioavailability of crude oil in aerobiosis. Crude oil viscosity and wax appearance temperature (temperature at which paraffin crystalizes) also decreased throughout the experiment[86]. This was attributed to the preferred degradation of long-chained hydrocarbons by the bacteria under both aerobic and anaerobic conditions. While BSF can contribute to crude oil mobility, the metabolization of long-chain alkanes can help eliminate wax from the reservoir and production equipment, increasing oil fluidity and reducing operation costs and equipment damage. In anaerobic flooding experiments with *G. pallidus* H9 in sandstone packed columns, previously subjected to an initial EOR by water flooding, 7% of added oil was recovered after a second water flooding, corresponding to the MEOR[86]. Although isolated from oil well injection water, *B. mojavensis* JF-2 did not use crude oil as substrate, utilizing instead water-soluble substrates and it was not inhibited by PHs. These characteristics, in addition to its ability to grow and produce BSF under wide ranges of temperature, pH and salinity, represent major advantages of this strain as a BSF producer for MEOR applications[41]. In anaerobic core displacement experiments at high pressure (7 MPa), *in situ* growth of *B. mojavensis* JF-2 resulted in a recovery of 23% of residual oil which was attributed to BSF production[99]. In the same experiment, a mutant unable to produce BSF resulted in only 6% oil recovery. *B. mojavensis* JF-2 has been patented[127] and tested with some success in a field trial[49]. *P. aeruginosa* SG was isolated and characterized with the aim of being used in *in-situ* BSF production MEOR strategies. In an anaerobic core flooding test mimicking reservoir conditions, a flooding inoculated with *P. aeruginosa* SG increased oil recovery by 8.33% of initial oil, resulting in a total recovery of 63.94% of all initial oil[81]. In a simulated MEOR anaerobic laboratory experiment conducted at 70 °C, a mixed culture composed mainly of *Thermoanaerobacter sp.* was able to remove 12% of the total heavy oil from carbonate porous media. BSFs were produced by the bacteria during the experiment[89]. These results indicate that *in situ* MEOR strategies with BSF producers may be feasible in high-temperature (70 °C) and high pressure (14.2 MPa) reservoirs even in those with low available O_2 and with heavy oils, high viscosity and density.

One of the first *in situ* studies to present economic feasibility of a MEOR strategy involving production of BSF used the facultative anaerobes *Bacillus* RS-1 and *B. subtilis* subsp. *spizizenii* NRRL B-23049[51]. Bacteria were inoculated into two limestone oil wells together with a supplement broth containing glucose. In other two oil wells only the supplement broth was added and a fifth well was used as negative control. Information on the concentration of oxygen was not provided, although there was evidence of anaerobic metabolism in inoculated wells. While BSF producers were found in the injected fluid of all five wells, BSF producers physiologically similar to the *Bacillus* were the only BSF producers recovered from the two inoculated wells. BSF production was observed only in the inoculated wells but the concentration of *Bacillus* cells remained close to the initial values. The

results were interpreted as an indication that both *Bacillus* strains were metabolically active, due to the supplemented broth which favored their metabolism instead of that of indigenous bacteria. Overall the results were considered a success, with concentrations of BSF much higher than their CMC being produced in the inoculated wells. Inoculation with the BSF-producing strain caused an average increase of oil production of one barrel per day, and the effect persisted up to 7 weeks after inoculation. From an economic point of view it was estimated that the MEOR strategy in this study had a cost of $1.6 per barrel[51]. A subsequent study performed under the same conditions but with an increased volume of broth and inoculum added to the wells, confirmed the cost-effectiveness of the process[52].

BSF production in high temperature reservoirs has also been reported[125,126]. In a MEOR strategy involving injection of air-supplemented water mixture in an oil well in China with stratal temperature of 60 °C, BSFs were detected together with an increase in aerobic and anaerobic bacteria. After six months of treatment, the concentration of methanogens and SRB returned to initial values[125,126].

A study using genetic markers to search for endogenous BSF producers in several American reservoirs found surfactin/lichenysin producing *Bacillus* in 8 out of 9 wells[124]. While genetic markers for the production of rhamnolipids by *Pseudomonas* were also searched for, no BSF producing pseudomonads were detected. The closest relatives to the endogenous *Bacillus* were *B. licheniformis*, *B. mojavensis*, *B. sonorensis* and *B. subtilis* subsp. *subtilis*[124]. This together with other previously mentioned *Bacillus* isolated from these environments (Table 1.1, section 1.10) indicated that they are common BSF producers in oil reservoirs and pseudomonads, on the other hand, seem to be rather scarce.

Hydrogen sulfide, a product of SRB metabolism, is often present in oil wells[128]. This compound is responsible for oil souring and infrastructures corrosion[129]. Since sulfide is known to inhibit BSF production under reservoirs conditions, its presence is of particular concern for MEOR strategies involving *in situ* BSF production[79,107]. A strategy involving a co-culture has been proposed, where one bacterial strain removes sulfide while another, *P. aeruginosa* SG or WJ-1, is responsible for anaerobic BSF production under low sulfide concentrations[79]. An easier to control and simpler approach involved the genetically modified *P. stutzeri* Rhl which is simultaneously able to inhibit SRB growth, remove sulfide and produce BSFs under anaerobiosis and up to sulfide concentrations of 33.3 mg/L[107]. These strategies do not only inhibit SRB development in oil reservoirs by having the exogenous bacteria successfully outcompeting them for nutrients, but also achieve the removal of some of the sulfide already present at oil reservoirs, thus increasing the quality of oil, decreasing associated carrion problems and specially, improving of the efficiency of MEOR strategies involving

in situ BSF production[79,107]. In general, these results highlight the applicability of anaerobic BSF production in MEOR, but also the importance of using strains adapted to the reservoirs conditions for attaining increasingly efficient and cost-effective MEOR applications.

1.7.3. Production of gas hydrates

Gas hydrates are crystalline structures of similar appearance to ice and are constituted by trapped gas molecules surrounded by bonded hydrogens present in water. They are of particular interest for storing a great volume of natural gas per volume of gas hydrate and are found in the subsurface of the ocean-floors. BSFs, independently of their type, have been found to lower induction time and increase formation rates of gas hydrates in the ocean-floor[130]. Anionic BSFs have been found to work as catalysts in the formation of gas hydrates by decreasing induction time, increasing formation rates, promote crystallization and not being consumed in the process. This results from the attraction of water molecules to the hydrophilic moiety of BSFs and the hydrocarbon gases to the hydrophobic part. Therefore, both compounds are brought together and induction time is reduced[28]. The formation of micelles may also aid the formation of gas hydrates[28,130]. Small amounts (ppm range) of the BSF influenced gas hydrates formation[130]. BSF producers, namely *P. aeruginosa* and *B. subtilis*, have been found in anaerobic mud of the gas hydrate stabilizing zone[131]. To test the effect of bacterial BSFs on the formation of gas hydrates in the ocean sediments, Zhang *et al.*, (2007) tested anaerobic surfactin in sand packed columns. The surfactin produced by *B. subtilis* under anaerobic conditions was much more effective in catalyzing the induction and formation of gas hydrates than aerobic-produced commercial surfactin, with an increase of 216% of formation rate[28]. This was attributed to a difference in isoform mass proportions in both BSFs, with some isoforms, more predominant in the anaerobic BSF, presenting a stronger catalytic effect. The morphology of the gas hydrates was also shown to be affected by the BSF used. Large nodular hydrates developed in the presence of the anaerobic-surfactin, while small grainy hydrated developed in aerobic-surfactin experiments[28].

1.7.4. Industrial BSF production and other industrial applications

During industrial production of BSF, oxygenation and agitation, used to enhance oxygen transfer rate, usually cause severe foaming. This results in problems of overflowing and contamination of tanks or equipment adjacent to the bioreactor, by rising and spreading of foam. Mechanical anti-foam strategies may not always work because of the amount of foam, and chemical strategies may inhibit bacterial growth. Microaerobic or anaerobic conditions represent a valuable industrial alternative to reduce or eliminate the foaming problem and only few cases of problematic foaming under anaerobic conditions have been reported[75,132–135].

BSFs are used in industry for their antiadhesive, antimicrobial and emulsifying properties. However, BSF are relatively expensive. A possible strategy to cut production costs is to use by-products as substrate for BSF production[136]. BSF can also be used in industry to promote the degradation of unwanted wastes and production of value-added products. In a study involving production of methane by anaerobic biodegradation of lipids from milk-fat, the synthetic surfactant sodium dodecylbenzenesulfonate (SDS) was added as an emulsifier[137]. Since SDS can be toxic to the fermentative bacteria only small concentrations are tolerated[137]. BSF are known to be more tolerated, thus their use instead of surfactants would likely increase production of compounds of interest. The addition of a BSF producer, instead of a synthetic surfactant, is also expected to lower production costs and reduce the need for continual addition of surfactant.

1.8. Conclusion

Aerobic BSF production is widely documented in scientific literature. However, the demonstration that bacteria able to produce BSFs in aerobic conditions may not be able to do it in anaerobic conditions and even if they are, the resulting BSF may be chemically and functionally different from that produced aerobically, has led to a growing interest in the understanding of the biological processes underlying anaerobic BSF production.

1.8.1. Biosurfactant producers

Most of the known BSF producers under microaerobic or anaerobic conditions are NRB, mainly belonging to the *Bacillus* and *Pseudomonas* genera. Anaerobic BSF production was reported to be represented in other genera of NRB, namely, *Bretibacillus, Rhodococcus* and *Geobacillus*. Regarding other metabolisms, so far, only eight bacteria using organic compounds as TEAs, belonging to *Bacillus*, *Clostridium*, *Thermoanaerobacter* and *Anaerophaga* genera, and two SRB, *Desulfovibrio* and *Thermoanaerobacter*, have been identified as being able to produce BSFs under oxygen-limiting conditions. It is not clear if NRB are the predominant group of bacteria able to produce BSF under anaerobiosis and microaerobiosis or if this fact is the result of an increased focus in the search of BSF-producing NRB and comparatively few studies regarding SRB, fermentative bacteria or bacteria using other TEAs. Despite many of the bacterial strains being isolated from oil, they have also been found in other environments, such as soil and sewage sludge. This indicates that bacteria able to produce BSF under oxygen limitation are ubiquitous even if present in low concentrations or with little diversity. However, to the best of our knowledge, many bacteria able to produce BSFs under aerobic conditions or isolated from anaerobic and microaerobic environments are never tested as possible microaerobic or anaerobic BSF producers. Therefore, it is reasonable to

speculate that this represent a small fraction of the total diversity of bacterial populations able to produce BSF under microaerobic and anaerobic conditions.

1.8.2. Biosurfactants produced in oxygen-limiting conditions

BSFs produced in microaerobiosis or anaerobiosis have been identified as rhamnolipids, surfactins, lichenysins A and B, iturins, fengycin and glycolipids. Rhamnolipids are produced by *Pseudomonas,* an unspecified glycolipid was detected in *Rhodococcus* and all other identified BSFs are *Bacillus* metabolites. However most of the BSFs produced under oxygen-limiting conditions are still unidentified and uncharacterized, limiting the perspective for possible applications of these BSFs and the comparison between the performances of BSFs produced under aerobiosis and anaerobiosis. An enlargement of the pool of known anaerobically-produced BSF is expected in the near future.

1.8.3. Metabolic pathways involved in oxygen-limiting BSF production

Little is known about the metabolism of anaerobic or microaerobic BSF production, even though often BSFs with different characteristics are produced in either anaerobic or anaerobic conditions. Further transcriptome and proteome studies should be performed to identify possible causes and differences between metabolisms in both conditions. Furthermore, genetic approaches such as mutagenesis may help identify relevant genes associated to BSF production in oxygen-limiting conditions.

1.8.4. Future perspectives

Considering the small number of isolates obtained so far and the scarcity of information on anaerobic or microaerobic bacterial BSF production, future research should focus on the prospection for new isolates and their characterization. Research on growth conditions and on the metabolic pathways involved in BSF production under oxygen-limiting conditions will also prove fundamental for maximizing the application of these bacteria in industrial and environmental contexts.

In general, comparing aerobic and anaerobic conditions, the latter are associated with longer lag times and slower growth. Additionally, biomass and BSF yields are also usually lower. Nonetheless, optimization of cultivation condition may reduce BSF production costs, making their applications economically viable. Likewise, understanding the interplay of factors affecting BSF production is also vital for the success of some of the more promising applications of anaerobic BSF producers, namely bioremediation or MEOR strategies.

Anaerobic or microaerobic BSF producing bacteria have been successfully applied in bioremediation and MEOR strategies and their potential for production of BSF and gas hydrates has

been demonstrated. Because of the differences in the BSF produced by the same strain under different oxygen conditions it is also possible that further investigation of facultative anaerobes or anaerobes able to produce BSF may result in an increase in the variety of available BSF, opening new perspectives for their applications.

1.9. Acknowledgments

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1.10. Tables

Table 1.1 – BSF producing bacteria under microaerobic or anaerobic conditions and respective BSFs characteristics. NA: not available. *: Values corresponding to each BSF.

Bacteria	Biosurfactant	Maximum decrease of surface tension in medium (mN/m) (aerobic values)	Maximum concentration of BSF produced (mg/l) (aerobic values)	Emulsion	Biofilm	Inoculum source	Reference
Anaerophaga thermohalophila Fru22 (DSM 12881)	Unidentified	25.9	NA	Yes	NA	Oil sludge and contaminated sedimentary residues from an oil separation tank; Hannover, Germany.	[83,84]
Bacillus amyloliquefaciens S499	Surfactin, iturin and fengycin	NA	252/108/10 (628/321/272)*	NA	Yes	Soil from Ituri, Congo	[61]
Bacillus licheniformis BAS50	Lichenysin A	≈20(26)	≈50 (155)	NA	NA	Oil from a reservoir at 1480 m depth; northern Germany.	[39,50]
Bacillus licheniformis BNP29	Unidentified	NA	≈35 (125)	NA	NA	Oil from a reservoir at a depth up to 1500 m; northern Germany.	[50]
Bacillus licheniformis BNP36	Unidentified	NA	≈8 (65)	NA	NA	Oil from a reservoir at a depth up to 1500 m; northern Germany.	[50]
Bacillus licheniformis Mep132	Unidentified	NA	≈45 (145)	NA	NA	Oil from a reservoir at a depth up to 1500 m; northern Germany.	[50]
Bacillus licheniformis VKM B-511	Unidentified	NA	NA	Yes	NA	Unknown	[54]
Bacillus mojavensis JF-2	Lichenysin B	≈41 (38)	34(110)	Foam	NA	Well injection water; Oklahoma, U.S.A.	[38,41–43,106]

Bacteria	Biosurfactant	Maximum decrease of surface tension in medium (mN/m) (aerobic values)	Maximum concentration of BSF produced (mg/l) (aerobic values)	Emulsion	Biofilm	Inoculum source	Reference
Bacillus strain RS-1 and *Bacillus subtilis* subsp. *spizizenii* NRRL B-23049	Unidentified	≈17 (in reservoir)	≈350	NA	NA	Sahara desert, Tunisia	[51]
Bacillus subtilis ATCC 21332	Surfactin	≈40	439 (45)	Foam	NA	Unknown	[28,55]
Bacillus subtilis BBG100	Mycosubtilin and surfactin	NA	≈70 (80)/≈20 (60)*	Foam	NA	Derivative of *B. subtilis* ATCC6633	[56]
Bacillus subtilis C9 (KCTC 8701P)	C9-BS	-44.6	4500(7000)	Yes	NA	Unknown	[60]
Bacillus subtilis strain #191	Unidentified	24.7	NA	Yes	NA	Crude oil; Brazil.	[26]
Bacillus subtilis strain #309	Surfactin	35	NA	Yes	NA	Crude oil; Brazil.	[26,53]
Bacillus subtilis strain #311	Surfactin	35.9	NA	Yes	NA	Crude oil; Brazil.	[26,53]
Bacillus subtilis strain #552	Unidentified	21.8	NA	Yes	NA	Crude oil; Brazil.	[26]
Bacillus subtilis strain #573	Surfactin	34.9	NA	Yes	NA	Crude oil; Brazil.	[26,53]
Bacillus subtilis subsp. *spizizenii* ATCC 6633	Mycosubtilin and surfactin	NA	45 (2)/≈90 (115)*	NA	NA	Unknown	[56]
Bretibacillus sp. BS2202	Unidentified	20.8 (22.7)	NA	NA	NA	Petroleum contaminated soil (30-40 cm deep); Noyabrskyi, Russia.	[76]
Clostridium pasteurianum sp.	Unidentified	NA	NA	NA	NA	NA	[85]

Bacteria	Biosurfactant	Maximum decrease of surface tension in medium (mN/m) (aerobic values)	Maximum concentration of BSF produced (mg/l) (aerobic values)	Emulsion	Biofilm	Inoculum source	Reference
Desulfovibrio desulfuricans subsp. *desulfuricans* El Agheila Z (DSM 1926)	Unidentified	21	NA	NA	NA	Sulphurous mud; Ain-ez-Zania lake, Lybia.	37
Geobacillus pallidus H9	Unidentified	35.48 (43.03)	NA	Yes	NA	Oil-containing brine; Daqing Oilfield, China.	86
Isolate Glc12	Unidentified	28.7	NA	Yes	NA	Oil sludge Petroleum contaminated sedimentary residues from a oil separation tank; Hannover, Germany.	84
Pseudomonas aeruginosa ATCC 10145	Rhamnolipid	≈28	≈100	Yes	NA	Unknown	74,103
Pseudomonas aeruginosa E03–40	Rhamnolipid	NA	≈4500	Foam	NA	Soil samples; Iowa, USA.	75
Pseudomonas aeruginosa PAO1 T	Rhamnolipid	NA	NA	NA	Yes	Unknown	73
Pseudomonas sp. ANBIOSURF-1	Rhamnolipid	≈41	NA	Yes	NA	Municipal sewage sludge; location unknown.	77
Pseudomonas sp. BS2201	Unidentified	14.5 (24.5)	NA	NA	NA	Petroleum contaminated soil (30-40 cm deep); Noyabrskyi, Russia.	76

Bacteria	Biosurfactant	Maximum decrease of surface tension in medium (mN/m) (aerobic values)	Maximum concentration of BSF produced (mg/l) (aerobic values)	Emulsion	Biofilm	Inoculum source	Reference
Pseudomonas sp. BS2203	Unidentified	17.7 (21.3)	NA	NA	NA	Petroleum contaminated soil (30-40 cm deep); Noyabrskyi, Russia.	[76]
Pseudomonas aeruginosa WJ-1	Rhamnolipid	(≈45)	390 (50200)	Yes	NA	Oil; Menggulin oil reservoir, China.	[79,80]
Pseudomonas aeruginosa PrhlAB	Rhamnolipid	-37.2	2420(17150)	NA	NA	Derivative of P. stutzeri SG, with plasmid pBBRPrhlAB.	[82]
Pseudomonas aeruginosa PoprAB	Rhamnolipid	-37.2	3560(20980)	NA	NA	Derivative of P. stutzeri SG, with plasmid pBBRPoprAB.	[82]
Pseudomonas aeruginosa SG	Rhamnolipid	32.97 (37.2)	1080(11650)	Yes	NA	Water sample; Xinjiang oil reservoir, China.	[79,81,82,104,107]
Pseudomonas stutzeri Rhl	Rhamnolipid	32.77	3120	Yes	NA	Derivative of *P. stutzeri* DQ1, with rhamnosyltransferase gene *rhlABRI* from *P. aeruginosa* SQ6.	[27,78]
Rhodococcus ruber Z25	Unidentified	NA	NA	Yes	NA	Formation brine; Daqing Oilfield, China.	[87]
Thermoanaerobacter pseudethanolicus 39E (ATCC 33223)	Unidentified	NA	NA	Yes	NA	Hot springs microbial mat; Yellowstone National Park, U.S.A.	[88]
Thermoanaerobacter sp. strains (mixed cultures)	Unidentified	NA	NA	NA	NA	Oil from carbonate reservoirs; Veracruz, Mexico.	[89]

Table 1.2 – Conditions for BSF production by BSF-producing bacteria under oxygen-limiting conditions. NA: not available. *: Values relative to biomass production (effect on BSF production was not tested/reported).

Bacteria	Special nutritional requirements	Carbon source	Anaerobic terminal electron acceptor	Oxygen conditions for production of BSF	Temperature (°C) (optimal)	Salinity (g/l) (optimal)	pH	Reference
Anaerophaga thermohalophila Fru22 (DSM 12881)	Hexadecane	Glucose	Organic compounds	Anaerobic	37-55	20-120 (≈ 60*)	6.7-6.8	[83,84]
Bacillus amyloliquefaciens S499	NA	Glucose, fructose, maltose, ribose, citrate, succinate, malate and casamino acids	NO_3^-, organic compounds	Aerobic, anaerobic	28-30	0	6.8	[61]
Bacillus licheniformis BAS50	$NaNO_3$	Glucose, sucrose	NO_3^-	Aerobic, anaerobic	25-50 (40-45)	0-130 (50)	5.4-8.5	[39,50]
Bacillus licheniformis BNP29	$NaNO_3$	Sucrose	NO_3^-	Aerobic, anaerobic	25-55 (45-50)	0-130 (50-70)	6.0-6.7	[50]
Bacillus licheniformis BNP36	$NaNO_3$	Sucrose	NO_3^-	Aerobic, anaerobic	25-55 (45-50)	0-130 (50-70)	6.0-6.7	[50]
Bacillus licheniformis Mep132	$NaNO_3$	Sucrose	NO_3^-	Aerobic, anaerobic	25-55 (45-50)	0-130 (50-70)	6.0-6.7	[50]
Bacillus licheniformis VKM B-511	Glutamic and aspartic acids	Glucose, sucrose, fructose, kerosene	NO_3^-	Aerobic, anaerobic	20-40 (30)	0-90 (90)	7.0-7.2	[54]
Bacillus mojavensis JF-2	DNA	Glucose	NO_3^-	Aerobic, anaerobic	40-50	20-100	4.6-9.0	[38,41–43,106]
Bacillus strain RS-1 and *Bacillus subtilis* subsp. *spizizenii* NRRL B-23049	Addition of a nutrient mix in oil wells	Glucose, sucrose	NO_3^-	Aerobic, anaerobic	28-37	50	7.4-7.6	[51]
Bacillus subtilis ATCC 21332	NA	Glucose	NO_3^-, NO_2^-	Aerobic, anaerobic	20-32	0-50	7.0	[28,55]
Bacillus subtilis BBG100	NA	Glucose	NA	Aerobic, microaerobic	30	0	6.0-7.0	[56]

Bacteria	Special nutritional requirements	Carbon source	Anaerobic terminal electron acceptor	Oxygen conditions for production of BSF	Temperature (°C) (optimal)	Salinity (g/l) (optimal)	pH	Reference
Bacillus subtilis C9 (KCTC 8701P)	NH_4HC0_3 and $MnSO_4$	Glucose, soybean oil, *n*-hexadecane	Organic compounds	Aerobic, microaerobic	30	0	6.8-8.0	[60]
Bacillus subtilis strain #191	NA	Tryptone, sucrose, *n*-alkanes	NO_3^-, Organic compounds	Aerobic, anaerobic	40-45 (up to 55 in solid medium)	10-100	7.0	[26]
Bacillus subtilis strain #309	NA	Tryptone, sucrose, *n*-alkanes	NO_3^-, Organic compounds	Aerobic, anaerobic	40-50 (up to 55 in solid medium)	10-100	7.0	[26,53]
Bacillus subtilis strain #311	NA	Tryptone, sucrose, *n*-alkanes	NO_3^-, Organic compounds	Aerobic, anaerobic	40-50 (up to 55 in solid medium)	10-100	7.0	[26,53]
Bacillus subtilis strain #552	NA	Tryptone, sucrose, *n*-alkanes	NO_3^-, Organic compounds	Aerobic, anaerobic	40-45 (up to 55 in solid medium)	10-100	7.0	[26]
Bacillus subtilis strain #573	NA	Tryptone, sucrose, *n*-alkanes	NO_3^-, Organic compounds	Aerobic, anaerobic	40-45 (up to 55 in solid medium)	10-100	7.0	[26,53]
Bacillus subtilis subsp. *spizizenii* ATCC 6633	NA	Glucose	NA	Aerobic, microaerobic	30	0	6.0-7.0	[56]
Bretibacillus sp. BS2202	P-limiting media with proteose peptone	Arabian light crude oil, glucose	NO_3^-	Aerobic, anaerobic	28	0-1	7.0-7.2	[76]
Clostridium pasteurianum sp.	NA	Sucrose	NA	Anaerobic	NA	NA	NA	[85]
Desulfovibrio desulfuricans subsp. *desulfuricans* El Agheila Z (DSM 1926)	NA	Na-lactate	SO_4^{2-}	Anaerobic	30	10	7.0	[37]

Bacteria	Special nutritional requirements	Carbon source	Anaerobic terminal electron acceptor	Oxygen conditions for production of BSF	Temperature (°C) (optimal)	Salinity (g/l) (optimal)	pH	Reference
Geobacillus pallidus H9	NA	Liquid paraffin, crude oil	NO_3^-	Aerobic, anaerobic	50-80 (65-70)	0-150 (<30*)	6.8-7.2	[86]
Isolate Glc12	Hexadecane	Glucose	Organic compounds	Anaerobic	37-55	20-120 (20-60*)	6.7-6.8	[84]
Pseudomonas aeruginosa ATCC 10145	P-limiting media	*n*-Hexadecane, palmitic acid, stearic acid	NO_3^-, NO_2^-	Aerobic, anaerobic	22-35	0.5-5	6.2-7	[74,103]
Pseudomonas aeruginosa E03–40	Betaine (osmopretectant)	Glycerol, tryptone	NO_3^-, NO_2^-	Aerobic, microaerobic	34	0.5	7.1	[75]
Pseudomonas aeruginosa PAO1 T	8% CO_2 (in anaerobic conditions)	Tryptone, glycerol	NO_3^- NO_2^-	Aerobic, anaerobic	30-37	0-10	7.0	[73]
Pseudomonas sp. ANBIOSURF-1	NA	Coconut oil	NO_3^-	Anaerobic	30	0-5	7.0	[77]
Pseudomonas sp. BS2201	P-limiting media with proteose peptone	Arabian light crude oil, glucose	NO_3^-	Aerobic, anaerobic	28	0-1	7.0-7.2	[76]
Pseudomonas sp. BS2203	P-limiting media with proteose peptone	Arabian light crude oil, glucose	NO_3^-	Aerobic, anaerobic	28	0-1	7.0-7.2	[76]
Pseudomonas aeruginosa WJ-1	$NaNO_3$	Glucose, paraffin, glycerol	NO_3^-	Aerobic, anaerobic	(37)	1	6.0-8.0	[79,80]
Pseudomonas aeruginosa PrhlAB	$NaNO_3$	Glycerol	NO_3^-	Aerobic, anaerobic	37	1	6.8	[82]
Pseudomonas aeruginosa PoprAB	$NaNO_3$	Glycerol	NO_3^-	Aerobic, anaerobic	37	1	6.8	[82]

Bacteria	Special nutritional requirements	Carbon source	Anaerobic terminal electron acceptor	Oxygen conditions for production of BSF	Temperature (°C) (optimal)	Salinity (g/l) (optimal)	pH	Reference
Pseudomonas aeruginosa SG	$NaNO_3$, PO_4^{3-}, SO_4^{2-}, Mg^{2+}	Glycerol, soybean oil, sunflower oil	NO_3^-	Aerobic, anaerobic	25-42	0-30	6.0-9.0	[79,81,82,104,107]
Pseudomonas stutzeri Rhl	KH_2PO_4	Glycerol, glucose, sucrose, molasses	NO_3^-	Aerobic, anaerobic	42	(1)	6.5-6.8	[27,78]
Rhodococcus ruber Z25	$NaNO_3$	Liquid paraffin, crude oil	NO_3^-	Aerobic, anaerobic	37	0	7.5	[87]
Thermoanaerobacter pseudethanolicus 39E (ATCC 33223)	NA	Glucose, tryptone	SO_4^{2-}	Anaerobic	65-70	12	NA	[88]
Thermoanaerobacter sp. strains (mixed cultures)	NA	Molasses	Organic compounds	Anaerobic	50-80	5-35	7.0	[89]

Table 1.3 – Characteristics of BSFs produced in microaerobic or anaerobic conditions. NA: not available.

Bacteria	Biosurfactant	CMC (mg/L) (aerobic values)	BSF activity tolerance to NaCl (g/l) (optimal)	BSF activity tolerance to temperature (°C)	BSF activity tolerance to pH (optimal)	Reference
Anaerophaga thermohalophila Fru22 (DSM 12881)	Unidentified	NA	NA	< 70 (20 min)	(2)	[83,84]
Bacillus licheniformis BAS50	Lichenysin A	12	<300 (< 100)	NA	NA	[39]
Bacillus mojavensis JF-2	Lichenysin B	10	50-100	30-50	NA	[38,41,43,49]
Bacillus subtilis C9 (KCTC 8701P)	C9-BS	40 mM	<58	20-100 (1 hour)	4.0-10.3 (5.0-9.5)	[60]
Bacillus subtilis strain #191	Unidentified	130	10-200 (50)	121 (20 min)	4-13 (6)	[26]
Bacillus subtilis strain #309	Surfactin	20	10-200 (50)	121 (20 min)	4-13 (6)	[26,53]
Bacillus subtilis strain #311	Surfactin	20	10-200 (50)	121 (20 min)	4-13 (6)	[26,53]
Bacillus subtilis strain #552	Unidentified	100	10-200 (50)	121 (20 min)	4-13 (6)	[26]
Bacillus subtilis strain #573	Surfactin	30	10-200 (50)	121 (20 min)	4-13 (6)	[26,53]
Geobacillus pallidus H9	Unidentified	22 (16)	NA	NA	NA	[86]
Pseudomonas sp. ANBIOSURF-1	Rhamnolipid	52	NA	NA	NA	[77]
Pseudomonas aeruginosa SG	Rhamnolipid	80	<150	4-121	2.0-10.0	[79]
Pseudomonas stutzeri Rhl	Rhamnolipid	90	<180	25-121	2.0-8.0	[27]
Rhodococcus ruber Z25	Unidentified	57	NA	NA	NA	[87]

Chapter 2

CHAPTER 2

Biodegradation of petroleum hydrocarbons in marine environments

Patrícia M. Domingues, Luísa S. Serafim, Newton C. M. Gomes, Ângela Cunha

2.1. Abstract

Release of petroleum to the environment can occur from natural sources, such as cold-seeps in the deep-sea, or anthropogenic sources, such as oil spills and transportation accidents. In these environments petroleum biodegradation by bacteria is a ubiquitous process. Here we discuss the fate of petroleum hydrocarbons in aquatic environments, and more specifically in submerged sediments, as well as the key players involved in petroleum biodegradation. Bioremediation is presented as a valid strategy to help remove the petroleum hydrocarbons from contaminated environments and the most well-known metabolic pathways involved in degradation of hydrocarbons are described. This work intends to summarize current knowledge on petroleum biodegradation under oxygen-limiting conditions, a process that is still less understood than its aerobic counterpart.

2.2. Key words

Crude oil; subsurface sediments; metabolic pathways; anoxia; hydrocarbon-degrading bacteria

2.3. Introduction

Petroleum, or crude oil, refers to naturally occurring mixtures of several hydrocarbons and, in smaller percentages, non-hydrocarbon compounds occurring in sedimentary rock deposits and which can be gaseous, liquid, semisolid or solid[138].

Petroleum hydrocarbon (PH) molecules occur in different structural forms, which have different terms according to chemists or petroleum geologists: *n*-alkanes or paraffins, iso-alkanes or iso-paraffins, cycloalkanes, also known as naphthenes or cycloparaffins, alkenes or olefins, and arenes or aromatics[138,139]. The first three are collectively known as saturated hydrocarbons. Aromatic hydrocarbons are unsaturated hydrocarbons that may also be linked to cycloalkane rings or alkyl chains[138]. The aromatic hydrocarbons fraction in crude oil can go from 1 to 20%, but is rarely above 15%, with the most common representatives being toluene and metaxylene. These together with benzene, ethylbenzene and other xylenes, make up the BTEX group, often used as indicators in bioremediation strategies due to their similar behavior in the environment. Polycyclic aromatic hydrocarbons (PAHs) are aromatics with two or more condensed rings, and can make up from 0.2 to 7 % of crude oil compounds[140]. The stability conferred by the benzene rings to the PAHs makes them

very recalcitrant in the environment and since several are known to be toxic, carcinogenic and mutagenic they are considered to be the most detrimental components of crude oil to contaminated ecosystems[141,142].

Since the modern discovery of oil in 1859 that there has been an ever growing demand for fossil fuel, as a result of a growing global population and economy and increased standards of living. Accidental and intentional releases of petroleum and its refined products are inevitable considering how extensively they are used as energy source and raw materials in almost all industries[138,140]. These releases occur in all types of environmental matrixes, like the atmosphere and soil. However, this work will focus mainly on the presence of PHs in water and aquatic sediment environments. As such, this work aims to describe and summarize the fate of PHs in marine environments. More specifically it will focus on PHs natural biodegradation, the bacterial communities involved, particularly under anaerobic conditions, and their metabolic pathways associated with hydrocarbon degradation. The potential for implementation of bioremediation strategies is also discussed.

2.4. Petroleum contamination and oil bioremediation approaches

2.4.1. Fate of petroleum and its products in oil spillages

PHs are ubiquitous in aquatic environments. Natural sources include diagenesis of sediments rich in organic matter, seeps of oil and natural gas deposits and biogenic hydrocarbons produced by benthos. Most anthropogenic sources are related to the petroleum industry, transportation activity, accidents, and municipal and industrial wastes[143,144]. Higher concentrations of PHs in costal environments are usually found near anthropogenic point sources indicating its main source as being human activity[145,146].

As soon as PHs are released into the environment they are subject to weathering processes, such as evaporation, emulsification, dissolution, dispersion, sedimentation, chemical oxidation and microbial biodegradation[138,140]. These processes lead to alteration on the composition of the hydrocarbons throughout time and may alter the toxicity of the contaminants and their biodegradability, furthermore they are involved in transporting the PHs away from the contamination source[140,147]. Weathering rates are variable and the highest rates are observed immediately after contamination[140]. They are conditioned by several environmental factors, such as, temperature, oxygen availability and moisture[138]. Biodegradation in particular is also affected by the indigenous microbial population and the composition of the contaminating petroleum or its products. The latter is known to be the main factor involved in the biodegradation rate and extent, with some hydrocarbons, such as strait-chained alkanes, being easily degraded, while others, like cycloalkanes with several rings, are more recalcitrant[138]. Furthermore, weathering usually leads to a reduction of

the monoaromatic hydrocarbons (MAHs) fraction and an increase in the impact of PAHs in overall toxicity[147].

Bioavailability describes the accessibility of a certain compound, in this case PHs, to organisms and the ability of these compounds to interact either by biological, physical or chemical processes with the organisms and the environmental matrixes[148]. Processes affecting bioavailability include dissolution, adsorption to solids or interfaces and absorption into cells or other porous particles[140]. Not all PHs present in marine environments are bioavailable, especially since most are present in an organic phase that is immiscible with the water column or sediments where most organisms are found.

Many PHs, specially compounds with a boiling point below 250 °C such as some aromatic hydrocarbons and *n*-alkanes with shorter chains than C_{14}, are dispersed, dissolved and evaporated[140,149,150]. Despite the action of weathering processes, PHs can reach marine sediments through sinking and sedimentation processes. During sinking, droplets of crude oil or some of its fractions that incorporate small sediment particles are transported downwards along the water column because of the higher density. In sedimentation, small amounts of oil components are sorbed to suspended sediment particles, for example, by being ingested by marine organisms and released as feces, which will eventually reach the seabed. Whether PHs reach the seabed by these processes depends on oil-to-water density ratios, currents and sediment interaction[140,151]. These processes are also responsible for the formation of oil conglomerations called marine tar residues, such as tar balls, which when not ashore, can remain in the seabed for long periods of time[152]. This may be of particular importance due to the possibility of recontamination of shores[153] but it has also been noted that while tar residues remain in sediments they represent lower toxicity to macro benthos than when they are suspended and dispersed[154].

The fate and persistence of PHs in either chronically or acutely contaminated environments is dependent on several complex weathering processes that can occur at different times and places[155]. Predominance, rates and duration of the processes vary depending on several parameters such as the type of contaminated matrix, such as water or sediment, and chemical and physical properties of the crude oil or petroleum product spilled[140]. As such, each spill case is very specific thus requiring tailored remediation strategies.

Both acute and chronic releases of PHs lead to environmental damage, which can present effects from short- to long-term. Acute releases are usually the result of accidents, such as in drilling and shipping[140]. These usually suffer large media coverage and attract public and scientific attention, with the Deepwater Horizon (DWH) off-shore drilling platform explosion and subsequent oil spill in 2010 being one of the latest and largest[156]. It should be noted that overall, there has been a decrease

in the amount of oil released by oil spills thought time. This is due to stricter legislation and improved ship and operations design[140]. Chronic releases of PHs are normally related to natural sources, such as natural seeps, leaking pipelines, presence of marinas or harbors, industrial and municipal wastewater discharges and receive much less attention. In other cases PHs are accumulated in submerged sediments, in which weathering processes are slow and so chronic release of almost fresh oil to the hydrosphere can occur[140]. Furthermore natural seepage of petroleum is thought to be responsible for at least half of the input of PHs in marine environments[140,157]. In most cases, PHs seepage in the deep-sea is a slow process, which allows for the native bacterial communities of these regions to adapt to their presence by either becoming tolerant to PHs or using them as carbon sources[158,159].

The main acute effect of PHs on animals is increased mortality, while sublethal effects, which may result from prolonged exposure and ultimately lead to reduced fitness, include diminished growth, development and reproductive rates and overall increased proneness to diseases[142,160,161]. These effects can last generations and involve many members of the trophic chain, disrupting the structure and function of the population, communities and ecosystems present in the contaminated areas[142,144,150,160]. The acute toxicity of an individual PH is often related to their solubility and narcotic effect, with aromatics and low molecular weight volatile compounds being the most influential. While the toxic effects of many individual compounds present in petroleum and its products are known, there is still little information regarding synergetic effects between hydrocarbons or interaction with other contaminants[140].

The impact of PHs on the environment depends little on the amount released and more on the rate at which it is released, the composition of the hydrocarbon mixture, and physical and biological characteristics of the contaminated areas and ecosystems[140]. Furthermore, contamination of shallow and inshore waters, like estuaries, has stronger effects on the environments than when contamination occurs in open sea[162,163]. Higher levels of suspended particles enhance aggregation thus reducing dispersion of the PHs and resulting in increased concentrations of contaminants. The PHs aggregated to the suspended particles eventually sediment at the bottom, and can remain there for long periods of time. However, disturbance of these sediments can release the PHs resulting in a recontamination[151,153,164]. Low molecular weight PAHs are dissolved in water while larger molecules are predominantly found in suspended solid matter and sediments[146,165]. Aromatic compounds with medium and high molecular weight, like alkylated phenanthrenes, are known to be the most persistent compounds in sediments[165,166] and animal tissues[167,168]. Therefore bioremediations strategies should take these aggregates into account in the restoration of contaminated estuarine environments[169]. In the particular case where the source of the spill is submerged and at considerable depths, as it happened in the DWH oil spill, the water soluble fraction of oil and gas tend to remain

longer in the hydrosphere instead of immediately starting to evaporate to the atmosphere as it happens in surface marine spills[170,171], potentially leading to stronger impacts in aquatic ecosystems.

2.4.2. Petroleum biodegradation and bioremediation approaches

In events involving a fast and/or extensive release of petroleum or PHs to the environment, like in the cases of the DWH drilling accident and the Prestige tanker sink, substantial negative impacts on local environmental quality and economy are expected especially when the oil reaches the coast. This affects mainly the local ecosystems and related activities like tourism, fishing, agriculture, industries and human health[169,172–174]. Therefore, prompt actions to control damage and remove or neutralize as much as possible the contaminants from the affected areas are required. It is for such situations that preventive measures must be placed and strategies predetermined by the competent authorities depending on the characteristic of certain areas.

Remediation strategies designed for the removal of PHs from aquatic environments can employ one or several physical, chemical or biological technologies[175] which are usually applied *in situ*[176]. The main processes used for reduction of PHs load in water are combustion, physical removal and containment of the oil, addition of chemical dispersants and bioremediation[152,177]. Strategies used in contaminated sediments include flotation and washing, coal agglomeration, thermal desorption, ultrasonic desorption, extraction using ionic liquids, chemical oxidation and bioremediation[175].

Bioremediation methodologies take advantage of the ability of organisms to degrade contaminants. In the case of PHs, bioremediation strategies employ organisms, mainly bacteria, fungi or plants, which are able to immobilize the contaminants or degrade and ultimately convert the hydrocarbons to CO_2 or to less toxic compounds[176]. However degradation may be incomplete and intermediary metabolites may be more toxic to the bacterial communities than their precursors thus hampering further biodegradation and increasing toxicity levels of the contaminated environment[175,178,179]. Bioremediation strategies can be reliable and efficient, used in large scale and are usually more affordable and environmentally safer than other alternatives. However, their success is dependent on several factors such as composition and characteristics of the PHs, environmental conditions and metabolic competence of the biodegradation agents[175,180].

Biostimulation and bioaugmentation are two of the most used approaches in petroleum remediation. Biostimulation consists on addition of nutrients, electron acceptors, or other compounds, such as surfactants, or also on alteration of environmental parameters, such as pH or temperature, so indigenous degrading organisms are stimulated increasing biodegradation rates[181–184]. Bioaugmentation involves the introduction of organisms, such as bacteria, fungi or plants, able to degrade PHs or to produce metabolites that increase biodegradation rates in the contaminated

environments[185–188]. Since bioaugmentation potentially reduces the adaptation time of the environment to the contaminant due to the introduction of organisms already adapted to hydrocarbon degradation, faster biodegradation rates have been found in bioaugmentation strategies when compared with biostimulation[189]. However biostimulation is often considered to be the most effective strategy since in some cases where exogenous organisms have been used, bioaugmentation strategies have been unsuccessful due to inadaptability to the environment or competition with indigenous organisms[190,191]. A proposed alternative is autochthonous bioaugmentation in which single strains or consortia composed of indigenous hydrocarbon degraders previously isolated from the affected environments are stimulated in laboratory and then added to the contaminated environments[189,191]. Besides solving the previously mentioned issues, this strategy has the added advantage that no foreign organisms are added to the contaminated environment[191]. Other alternatives involve adopting more inclusive bioaugmentation strategies like specific and environmentally-tested consortia composed of degraders and non-degraders, or genetic bioaugmentation strategies, consisting in the introduction of a microbial host carrying a plasmid with catabolic genes which can be passed via horizontal transfer to the autochthonous population conferring it the ability to degrade PHs[192].

In most cases, natural attenuation of PHs is limited by inorganic nutrients, mainly nitrogen and phosphorus, which in general, are present in low concentrations in seawater and crude oil[181,183,193]. Therefore, an effective biostimulation strategy requires the addition of these nutrients to petroleum contaminated areas[182,183,194]. However, amendments must be carefully thought-out as to avoid undesirable effects, such as eutrophication and inhibition of PH-degradation[183,195]. Nutrient concentration influences the composition of bacterial communities which in turn, affects the proportions of degradation of aliphatic and aromatic hydrocarbons[182,183,196].

The bioavailability of PHs is also an important parameter that can limit oil biodegradation[197]. The use of chemical dispersants, in which the main active compounds are surfactants, is considered to be one of the few methods applicable in large oil spills with proven results that reduce environmental and economic impacts of oil contamination[177,198,199]. The principle behind the usage of chemical dispersants is to reduce interfacial tension between oil and water, so that micelles are formed that trap PHs, altering their solubility and volatility, and increasing the surface area between the two phases. This enhances pollutant bioavailability, and stimulates weathering processes, such as chemical and microbial oxidation and dissolution, reducing the contaminants concentration in the environment[199–201]. However, chemical dispersants themselves have been associated with environmental and human toxicity[173,198,202,203]. These compounds can be replaced by biosurfactants (BSFs), which are biologically produced molecules with the same surface-active properties as surfactants but often presenting lower toxicity and higher biodegradability[23,204]. Many bacteria are equipped with coadjutant mechanisms, such as BSF production, that increase PHs bioavailability. In

bioremediation strategies, besides the direct addition of BSF to the contaminated areas, it is also possible to apply bioaugmentation with BSF-producing or with BSF-producing PH-degrading bacteria[32,205,206]. For optimal results, BSF-producers for bioaugmentation strategies should be chosen not only in function of their ability to grow under the prevailing environmental conditions, but also considering the properties of the BSF and the effects on autochthonous bacterial community.

Biodegradation of crude oil, either in aerobic or anaerobic conditions, mainly results in a decrease of the aliphatic and aromatic hydrocarbon fractions and an increase in the non-hydrocarbon and more polar fractions (Figure 2.3)[197]. In the first days after petroleum contamination low molecular weight aliphatic and aromatic hydrocarbons are degraded. During this stage, blooms of the alkane-degrading specialists, such as *Alcanivorax* spp.[207–209], typically occur. During the first month and usually during or following the bloom of alkane-degraders, a bloom of aromatic hydrocarbon specialists, such as *Cycloclasticus* spp., develops[209–212]. However there have been instances where other less common PH degraders form blooms[210,213], indicating that different bacterial communities can reach the same levels of bioremediation and be used effectively in bioremediation strategies[197]. In general, the most easily degraded PHs are saturated alkanes, followed by low molecular weight aromatic compounds, branched alkanes, aromatics with higher molecular weight, cycloalkanes and finally polar compounds. More soluble compounds with larger surface area are more bioavailable to bacteria, thus more prone to biodegradation. On the other hand, complex and very stable structures, such as PAHs and high molecular aliphatic hydrocarbons, are harder to be metabolized and can take weeks to years to be removed from the environment[140,165,170,212]. While the

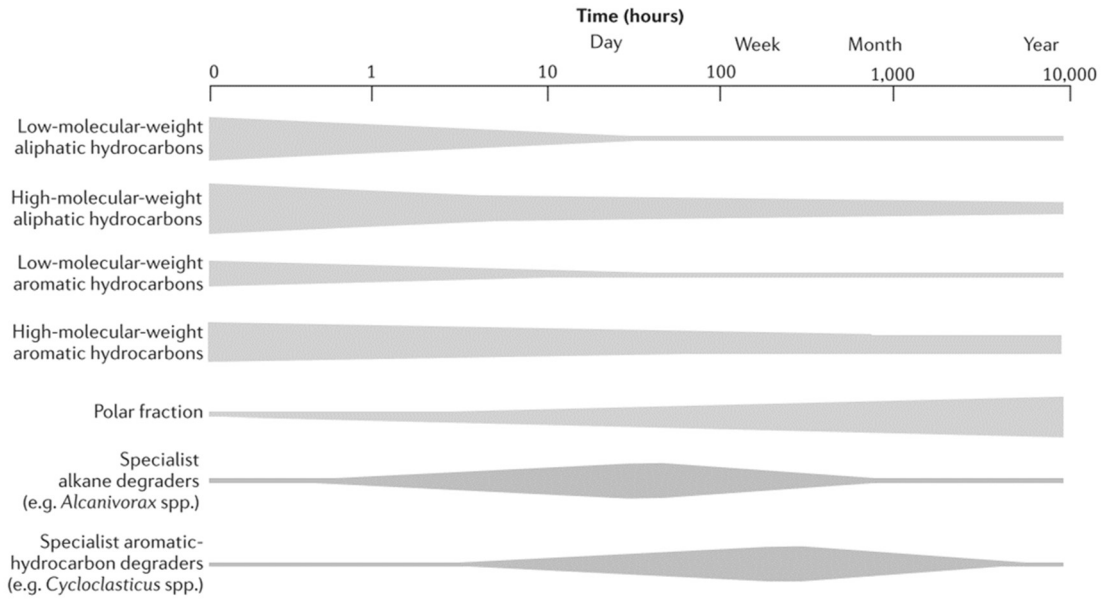

Figure 2.3 – Generic representation of the abundance over time of the different PHs fractions and appearance of blooms of PH-degrading specialists in oil spills[197].

aliphatic fraction may be predominant, it is also the most degraded. Thus, some the more recalcitrant and toxic aromatic hydrocarbons and non-hydrocarbon compounds present in crude oil have a stronger impact on the environment[197]. Addition of smaller or more easily degraded hydrocarbons, which are usually biodegraded first, has been shown to accelerate the start of biodegradation of more complex PHs[170,214]. Previous exposure to PHs has been found to reduce the response time of the indigenous bacterial communities able to perform biodegradation without affecting biodegradation rates[215]. These appear to be mainly related to the PHs concentration, with biodegradation rates decreasing and biodegradation eventually stopping when low concentrations are reached[216].

Biodegradation of PHs by indigenous bacteria present in contaminated environments is a ubiquitous process. However, the efficacy of the natural process and the preference for certain compounds varies with the location and structure of the bacterial community[217,218]. Indigenous hydrocarbonoclastic bacteria are usually present in small or residual quantities in marine environments. These communities quickly flourish upon petroleum or petroleum-based products contamination of the environment and PHs become available to be used as carbon and energy sources[209,217]. As such, PHs contamination imposes a strong selective pressure in bacterial communities[219]. This leads to a decrease of overall community diversity as a result of the toxicity of some PHs and the selectivity of specialized PHs degraders[219–223]. In most cases, bacterial biomass increases soon after contamination and communities shift over time, with certain bacterial strains predominating at certain points in time, depending on the presence and bioavailability of specific compounds[224,225]. Once a certain group of PHs is mostly depleted and other becomes predominant, the bacterial community adjusts and the previously predominant strain gives way to a new bloom of degraders of the next group of compounds. As such, spatial and temporal alterations to the composition and quantity of PHs will affect the succession profiles of the bacterial community [212,224,225]. Contamination with different PHs results in different composition of the consortia of hydrocarbon-degraders[220]. After extensive natural biodegradation, when PHs levels become closer to initial values, predominance of hydrocarbonoclastic bacteria is attenuated returning to minimal concentrations[209]. In general, bacterial diversity is inversely correlated with PHs concentration in contaminated environments[226].

Besides the amount and type of available carbon sources and nutrients, other factors like interactions between organisms[227], availability of terminal electron acceptors (TEAs)[184] and physicochemical parameters, such as, temperature[221,228], salinity[32,229,230] and pressure[231] affect bioremediation strategies. Optimization of these parameters can increase the potential success of the bioremediation strategy by acting as selective pressure for the development of PH-degrading bacteria adapted to the prevailing environmental conditions at contaminated ecosystems. This leads to shifts in composition and structure of microbial communities, which ideally, enhance the presence and

activity of PH-degraders[184,221]. The environmental matrix or matrices to be decontaminated can also affect bioremediation. For instance, while oil in the water column is usually quickly degraded, the fraction buried in sediments can be much more recalcitrant due to limited mass transport of hydrocarbons, nutrients or oxygen[197]. Biodegradation plays an important role in removing tar residues from marine sediments. However, biodegradation rates can be especially low in these cases since bacteria tend to concentrate in the oil-water interface, which can be proportionally a small area compared to the volume of the tar residue, and the components of weathered oil can be difficult to metabolize[152,232]. Tar residues are of particular concern since they can persist for several years or even decades in marine sediments, often under other layers of sediment effectively hiding them[233,234] making them difficult to identify so that bioremediation processes can be applied. In some instances, high energy processes, such as the action of waves or particularly violent storms, can remove the upper layers of sediment exposing and dispersing the submerged tar residues, effectively leading to a recontamination of the environment. However, this may also favor the clearance of tar residues from the environment by making them more accessible to biodegradation and physical removal processes[153,164,235,236]. As such, bioremediation strategies should be designed having these parameters into consideration.

To understand which hydrocarbonoclastic bacteria are of particular importance in PH bioremediation strategies and distinguishing them from non-degraders, culture-based studies are necessary to assess the catabolic abilities of the microorganisms, and environmental studies are needed to understand the *in situ* role of particular microbial groups[197]. Culture independent techniques are useful to determine and follow microbial community dynamics. These tools are of special interest to assess the response of the communities to different bioremediation treatments, to compare the results from *in situ* and microcosm approaches, and to predict the impacts of environmental conditions on bacterial communities[197].

Some of the bacteria involved in PHs bioremediation strategies are non-degraders[237–239]. Nonetheless these bacteria can also be fundamental for the success of the bioremediation strategies, often being responsible for bottlenecks in the biodegradation process, by controlling nutrients, metabolic intermediaries, or products fluxes and concentrations. In some cases the bioremediation strategies may be more successful by focusing on the stimulation of these bacteria[197,240].

So far, the best way to predict how PHs will impact and behave in a certain environment is through modeling tools. However, due to the complexity of parameters affecting each case, as mentioned in the previous section, site-specific models are necessary to develop response plans. Modeling requires both baseline data and information on responses to the presence of contaminants[140]. Since obtaining this information *in situ* is not always feasible, the utilization of

microcosms and mesocosms can be useful to predict *in situ* bioremediation under a controlled and more easily manageable environment. Most studies directed to responses of bacterial communities were obtained by these approaches[216,218,222,223,241,242] even if providing limited information, considering that they do not account for all the complexity, diversity and dynamics of natural environments[143]. In most recent studies, there is a trend to perform *in situ* evaluations by applying molecular techniques to characterize bacterial communities[209,243,244].

2.4.3. Bioremediation approaches and petroleum biodegradation under oxygen-limiting conditions

Most bioremediation strategies available at the moment are designed for aerobic conditions. However petroleum spill disasters can also occur and affect microaerobic or anaerobic niches. One of the best examples is the DWH oil spill, which occurred under water at a depth of approximately 1500 m[245]. This event tested the technical ability to implement oil spill mitigation actions. It is now recognized that the strategies available at the time were not thought out for the scale and nature of the DWH oil spill. As such, the paramount need to perfect and develop bioremediation strategies for environments lacking in O_2, that can be included in oil spills contingency plans, gained relevance[209].

Since investigation on anaerobic degradation of PHs is relatively recent, bioremediation of anaerobic environments is still a challenge and some strategies used in aerobic conditions are simply not applicable. In the last few years, new techniques and approaches to bioremediation of anaerobic sediments contaminated with PHs have been tested with some success. Most of these new approaches are biostimulation strategies, either of the anaerobic hydrocarbonoclastic bacteria[243,246] or by applying aeration techniques and stimulating aerobic hydrocarbonoclastic bacteria[209]. The major disadvantage of the first is that the time necessary to reach similar levels of biodegradation is much longer when using anaerobic hydrocarbonoclastic bacteria than aerobic hydrocarbonoclastic bacteria due to the low hydrocarbon degradation rates of anaerobic bacteria[209]. On the other hand, aeration of anoxic sediments involves extra equipment and makes the process more expensive and energy-demanding and, depending on the affected environment, it may not feasible due to the size or characteristics of the area involved[246]. In most instances of PHs contamination of microaerobic or anaerobic environments bioremediation is not even attempted, leaving the decontamination of the site to natural attenuation, or aerobic bioremediation strategies are employed instead. Therefore, there is a need to develop bioremediation strategies adequate to environments with oxygen-limiting conditions.

Anaerobic biodegradation approaches can vary in the time necessary to return the affected environments to pre-contamination conditions, depending on the physicochemical and

microbiological properties of the area affected, the composition and complexity of the contaminant and the techniques applied, among other factors. While aerobic biodegradation strategies may require no more than a few days[247], anaerobic strategies can take from a few weeks[248,249] to some years to be completely effective[166,243]. Complete bioremediation can require the application of both aerobic and anaerobic strategies, since PHs can migrate from aerobic to anaerobic environmental compartments, and aerobic environments, such as submerged sediments, can quickly become anoxic in the presence of the contaminants, due to an initial increase in oxygen consumption by aerobes stimulated from the sudden carbon source input[140,250].

Oxygen in an important factor to the success of bioremediation strategies as it is the most commonly used TEA in the biodegradation of PHs, which are highly reduced substrates. Oxygen is often the limiting factor in aerobic hydrocarbon-degradation[195] instead of nutrients[251,252], with aerobic degradation of PHs by aerobic bacteria being inhibited at oxygen concentrations below 0.013-1.5 $mg \cdot O_2/L$[253]. Oxygen is also an important selective driver of the composition of PH-degrading populations[228]. In microaerobic zones, very low concentrations of oxygen may be enough to promote biodegradation of more recalcitrant PHs by facultative anaerobic bacteria. Those recalcitrant PHs, cannot be degraded or take a long time to be degraded by anaerobic bacteria likely due to the necessity of hydrocarbons to be oxidized before uptake by anaerobes[74,103,253]. A metaproteomic and metabolomic study of chronically polluted environments demonstrated that low oxygen concentrations can have inhibitory effect on metabolic pathways associated with bacterial degradation of PHs[254].

An important bioremediation strategy to improve *in situ* bioremediation of oxygen deprived environments is the addition of alternative TEAs, such as those presented in Table 2.4, in order to stimulate more energetically favorable metabolic types involved in PHs degradation[169,255]. This is of particular interest in aquatic or submerged environments since most TEAs, such as sulfate and nitrate, used in anaerobic metabolism are more soluble than O_2[248,253]. TEAs receive electrons that are transferred from electron donor substrates (e.g. hydrocarbons) by bacteria, while generating energy necessary for cellular activity and growth in the process [256]. TEAs with higher standard reduction potential tend to be preferred by microorganisms over those with lower reduction potential, due to greater free energy released[35,256,257]. While many bioremediation studies have focused on the effects of the use and predominance of one specific TEA, mainly nitrate or sulfate, few studies have compared the effects of mixed TEAs. In an anaerobic study conducted in marsh sediments contaminated during the DWH incident, a mixture of nitrate and sulfate increased the degradation of

total hydrocarbons (78%) over a period of 80 days, when compared to sulfate-reducing (55%), nitrate-reducing (30%) and fermenting conditions (19%). Furthermore a higher number of colony forming units, in the order of 10^8 CFU/mL, was observed in mixed TEAs conditions than in the sulfate, nitrate and fermenting (10^7) and the natural attenuation control (10^4)[169]. This indicates that the simultaneous addiction of different TEAs increases bacterial abundance, especially of those groups directly and indirectly involved in PH degradation, leading to a more diverse and competent community. Addition of TEAs, besides leading to a shift in the structure of bacterial communities, has also led to an increase in the abundance of genes involved in hydrocarbon-degradation and associated to catabolic pathways (e.g. degradation of benzoyl-CoA)[184].

Table 2.4 – Known TEAs used by bacteria in PHs degradation, corresponding standard reduction potentials ($E^{0'}$; pH 7, 25°c) of redox couples and standard free energies ($\Delta G°$) of the corresponding metabolisms. *Sulfate reduction from methane. Adapted from [257], [357] and [525].

Environmental condition	**Aerobic**	**Anaerobic**				
Redox condition	Oxidized	Moderately reduced			Highly reduced	
TEA	O_2	Fe(III)	Mn(IV)	NO_3^-	SO_4^{2-}	CO_2
Product of TEA reduction	H_2O	Fe(II)	Mn(II)	NO_2^-	HSO_3^-	CH_4
Predominant metabolism	Aerobic	Facultive anaerobic			Anaerobic	
$E^{0'}$ of redox couples (mV)	+820	+772	+380	+430	-516	-240
$\Delta G°$ (kJ/mol)	-770	-697	-557	-463	-98/-33*	-57

Natural attenuation of PHs in anaerobic contaminated environments by several bacteria using different TEAs occurs even under low temperature[226]. This is important considering that many PH contaminated environments are located in cold climate zones.

Biological relations within microbial communities, including interspecies syntrophy involving hydrogen or formate, are important microbial interactions in anaerobic bioremediation strategies. This is of particular interest in methanogenic conditions, where alternative TEAs to oxygen are absent or present in limited amounts[227]. In general, syntrophic associations between

fermentative bacteria and methanogens comprise of two parts. Initially the fermentative bacteria metabolizes the available substrate, e.g. PHs, producing the methanogenic substrates hydrogen, formate and acetate. Finally, methanogens convert these products to methane and CO_2. This leads to a reduction in the concentration of the intermediary metabolites which allows for the overall degradation process to be thermodynamically favorable[258].

Considering that only a small fraction of bacteria are cultivable[259] and culturing strict anaerobes is a complex and time consuming process, in most anaerobic biodegradation studies molecular techniques are used to assess the structure of bacterial communities[176,241,246,254]. Some of the most popular approaches to bacterial diversity and bacterial community composition are denaturing gradient gel electrophoresis (DGGE), for molecular fingerprinting analysis[213,246,260,261], and gene cloning and sequencing[209,241,243]. Advanced sequencing methods, such as pyrosequencing, have recently started to be used in anaerobic bioremediation strategies as tools used in determining communities structure and bacterial function, specially following the DWH spill[159,262,263].

2.5. Hydrocarbonoclastic bacteria

Hydrocarbonoclastic bacteria are a group of bacteria that are able to use one or more PHs as carbon sources, often in detriment of other carbon sources[264–266]. This continuously growing group is composed of several genera belonging to Proteobacteria, Actinobacteria and Firmicutes phyla[197,215,223,267,268]. Degradation of PHs by hydrocarbonoclastic bacteria has been found in both aerobic and anaerobic environments. These bacteria are ubiquitous, although representing a minor fraction of the community in non-contaminated environments, blooms appear upon contamination with PHs[143,208,212]. These bacteria proliferate by taking advantage of substrates that are not used by most bacteria[267]. Some genera of known hydrocarbonoclastic bacteria are specialized in the degradation of certain PHs groups. *Alcanivorax* spp. or *Thalassospira* spp. specialize in the degradation of aliphatic hydrocarbons, while *Cycloclasticus* spp. or *Mesorhizobium* spp. are specialized in the degradation of aromatic hydrocarbons[223,269].

While most hydrocarbonoclastic bacteria are also able to use other organic substrates as carbon and energy sources, there is a ecophysiological group of bacteria, mainly from marine environments, that are known as obligate hydrocarbonoclastic and are solely specialized in metabolizing PHs[143]. These bacteria are ubiquitous in marine environments, having been detected in surface and deep seawater and submerged marine sediments. Known bacteria of this small yet important group belong mainly to genera *Alcanivorax*, *Marinobacter*, *Thallassolituus*, *Cycloclasticus*, *Oleispira*, *Planomicrobium*, *Yeosuana*[143], *Porticoccus*[270] *and Tamilnaduibacter*[271]. Some obligate hydrocarbonoclastic bacteria, such as *Cycloclasticus oligotrophus*, are known to have

a high affinity to certain hydrocarbons, thus being able to metabolize them even when present in low concentrations[240].

Submerged PH contaminated sediments have higher bacterial diversity and variability between different sampling sites than the seawater above. However more culturable hydrocarbonoclastic bacteria have been isolated from water than from sediment[261]. Oil reservoirs and associated infrastructures, such as storage tanks, are also common sources of aerobic and anaerobic hydrocarbonoclastic bacteria[272,273]. Most biodegradation in oil reservoirs occurs in water-oil interfaces where bacteria can easily interact with hydrocarbons[273]. Some of the PH-degrading strains isolated from these environments are extremophiles, presenting high resistance to high temperatures[274], salinity[32] and pressure[275] .

2.5.1. Anaerobic hydrocarbon-degrading bacteria

Research on aerobic biodegradation of hydrocarbons has been in course for over a century[276]. However, only since the late 1980s have researchers started investigating anaerobic degradation of hydrocarbons by bacteria[277]. Anaerobic bacteria can be facultative anaerobes or strict anaerobes depending if beside anaerobic life they are also able to use and tolerate oxygen or not, respectively[34,35]. Natural anaerobic biodegradation of PHs occurs mainly in oxygen-deprived environments containing PHs. That is the case of oil reservoirs, where biodegradation alters petroleum characteristics and affects the extraction process and its cost[278], the deep ocean near natural seeps[279,280] and most microaerobic or anaerobic environments contaminated with PHs[218,263,281]. Bacteria able to degrade hydrocarbons in oxygen-limiting conditions are ubiquitous in anaerobic environments, such as deep-sea water[282] and submerged sediments[283].

As with aerobes, degradation rates of facultative and strict anaerobes can be affected by several parameters, while in most cases they are the same there have been found some particularities. Short-chained alkanes (C_{11}-C_{13}), due to their inherent toxicity, present lower degradation rates in nitrate- and sulfate-reducing conditions than in aerobic conditions[248]. However, small concentrations of O_2 have been shown to enhance anaerobic biodegradation of PAHs when compared to strictly anaerobic conditions[186] and anaerobic hydrocarbon-degrading bacteria biodegradation rates are less affected by cold temperatures than aerobic hydrocarbon-degrading bacteria[228].

Due to O_2 limitation in microaerobic and anaerobic conditions, bacteria living in these environments operate metabolic pathways that require alternative compounds as TEAs. Most anaerobes use nitrate or sulfate as TEAs. However, some bacteria are able to grow in anaerobic conditions using other alternatives such as Fe(III), Mn(IV) or organic compounds. Different anaerobic metabolisms can be simultaneously responsible for PH degradation by different bacteria

in microaerobic and anaerobic environments, as long as the TAEs are available. Furthermore, the predominant metabolism may change throughout time and space, depending on the concentrations of specific TAEs, electron donors and variations of several parameters, such as pH[226,284].

Besides metabolic versatility in terms of organic carbon sources uptake, some bacteria are able to reduce several different TEAs. Therefore, these bacteria easily adapt to temporal and spatial fluctuations of TEAs and electron donors and can usually be found in environments where these changes are observed, like coastal sediments[263,282,285].

2.5.1.1. Sulfate-reducing bacteria

Sulfate-reducing bacteria (SRB) are characterized by their ability to use sulfate or other oxidized compounds of sulfur as TEAs in anaerobic respiration. Hydrogen or organic compounds are used as electron donors and sulfide is produced from reduction of sulfate. Most SRB are strict anaerobes, but some are considered aerotolerant[129].

Only recently was it established that biodegradation of PHs in anaerobic sediments is heavily influenced by SRB[129,218]. SRB are ubiquitous in anaerobic environments, being commonly found in submerged marine sediments, microaerobic or anaerobic zones of the water body (e.g. zones with high salinity) and oil reservoirs[129,286]. These bacteria are able to degrade aliphatic[243,248], MAHs[219] and PAHs ranging from low (2-3 rings) to high (>3 rings) molecular weight[218,219,287], and are particularly responsible for anaerobic degradation of BTEX[129]. Degradation of branched alkanes appeared to be preferred over linear alkanes, and long-chained alkanes over short-chained[248]. In marine sediments, SRB appear to be the most energy efficient, and therefore the predominant hydrocarbon degraders[219,268], in relation to Fe(III)-reducing bacteria (FeRB) that predominate in freshwater sediments[255]. Most likely because marine environments are rich in sulfate due to high concentration in sea water (≈28 mM), but can present limited concentrations of nitrate or Fe(III)[218,286]. However, low concentration of organic matter limits the activity of SRB as sulfate and carbon sources availability in the deep seafloor decrease with depth[286]. SRB play an important role in the carbon and sulfur cycles in the environments they are naturally found in.

Due to their wide degradative ability, SRB can be important agents of PHs biodegradation, even if this is usually a slow process that can take up several months or even years[218,243]. Given enough time indigenous communities of SRB from sediments of different marine harbors are able to degrade PAHs. However, communities from different sediments achieve different levels of biodegradation for different compounds[218]. In a field experiment in anoxic coastal sediments over the span of 503 days, two groups of hydrocarbonoclastic SRB, *Desulfococcus–Desulfonema–Desulfosarcina*-like and *Desulfovibrio–Desulfomicrobium*-like, were detected in both control and

petroleum-added areas. The relative abundance of each group, initially similar, was significantly affected by the addition of oil at the end of the experiment[243]. In a similar but shorter study, PHs-degrading or PHs contamination-associated *Deltaproteobacteria* were predominant and almost 10% of the clones obtain at the end of 90 days were related to *Desulfatibacillum aliphaticivorans*, a alkane and alkene-degrading SRB[209]. Several of the most abundant clones detected in both studies were related to known PH degraders. Analysis of coastal submerged sediments affected by the Prestige oil spill revealed that Deltapreoteobacteria were predominant, with *Desulfobacteraceae* being the most abundant group and SRB playing an important role in PAHs degradation[219]. Deltaproteobacteria family *Desulfobacteraceae* have also been detected in mesocosms of pristine coastal sediments experimentally contaminated with crude oil and naphthalene[222].

SRB have also been found in bacterial communities from cold seeps and mud volcano sediments, where small PHs (C_1-C_5), mainly methane, are expelled from the marine subsurface onto the hydrosphere. Here they take advantage of the expelled hydrocarbons, using them as carbon and energy source[279,288–290]. Anaerobic methane oxidation is a thermodynamically challenging process that often occurs from a symbiotic relationship between SRB and methanogenic Archaea. Several mechanisms for sulfate-dependent anaerobic methane oxidation have been proposed but further experimental confirmation are needed and the exact mechanism or mechanisms are still unclear[291]. Besides methane-degraders, other PH-degrading SRB belonging to the *Desulfosarcina/Desulfococcus* clade have been identified in these environments[280].

Because dissimilatory sulfate-reduction ultimately leads to the formation of sulfide, SRB are also known to be the cause of souring in oil reservoirs[128]. Since sea water has a high concentration of sulfate, sulfide produced by reduction of sulfate represents a problem in terms of toxicity and corrosion and it affects not only oil, but also the infrastructures present in oil reservoirs, especially in exploration facilities in marine environments[129,286].

2.5.1.2. Nitrate-reducing bacteria

Nitrate-reducing bacteria (NRB) are able to reduce nitrate to nitrite using the first as TEA while oxidizing organic matter, hydrogen, reduced iron or sulfur species. This is the first step involved in denitrification and dissimilatory nitrate reduction to ammonium process. The former culminates with the release of N_2 and the latter with ammonium[292]. NRB play a particularly important role in costal and estuary submerged sediments in the removal of the accumulated nitrogen from these environments[219,293].

Nitrate is one of the most thermodynamically favorable electron acceptor used under anaerobic conditions (Table 2.4)[35]. In such conditions, when nitrate is present in non-limiting

concentrations, NRB are often the predominant group in communities[184]. With the continuous search for new hydrocarbon-degrading bacteria new NRB able to degrade PHs are constantly being discovered and this diverse group is comprised by several different genera. The most commonly identified and well described hydrocarbon-degrading NRB representatives belong to *Thauera* and *Azoarcus* genera, known to degrade several aromatic hydrocarbons[184,185,242,272,294–296]. Up until 2012, all known hydrocarbon-degrading NRB had been identified and isolated from terrestrial and freshwater environments, with no evidence existing of the presence of hydrocarbonoclastic NRB in marine environments. More recently it has been noted that while most freshwater and terrestrial hydrocarbon-degrading NRB belong to the *Betaproteobacteria* class, isolates from marine environments belong mainly to *Alpha-* and *Gammaproteobacteria* classes. Furthermore marine hydrocarbonoclastic NRB communities appear to be more diverse than those from freshwater sediments[144].

Molecular and culture based techniques have been employed in isolating and identifying several denitrifying bacteria associated with PHs biodegradation in chronically polluted sediments[242,260]. Overall, a great variety of hydrocarbonoclastic NRB and denitrifying bacteria has been reported. A study conducted with coastal sediments showed that most NRBs belonged to the *Gammaproteobacteria* class and that due to the versatility of the community, the addition of crude oil to the enrichment cultures had little influence over its structure[242]. Unlike a similar field study with SRB where the communities structure were strongly affected by the addition of crude oil by the end of the experiment[243].

Other sources of PH-degrading NRB are crude oil and other matrixes related to the petroleum industry. Numerous isolates, mainly *Bacillus* sp. and *Paenibacillus* sp., able to degrade several hydrocarbons and produce BSFs have been identified[26,272]. Furthermore, stimulation of NRB in oil reservoirs, by addition of nitrate or allochthonous NRB, is a common strategy to inhibit SRB growth and souring of oil[297].

In anaerobic biostimulation strategies taking advantage of PH degrading NRB, nitrate is often added to the contaminated environments. It has been proved that this causes an increase in the number of genes linked to hydrocarbon-degradation, mainly those involved in central catabolic pathways of aromatic hydrocarbons degradation originating from NRB, thus increasing PH degradation and nitrate-reduction[184].

Facultative anaerobic NRB can be of particular interest for biodegradation of PHs in microaerobic environments, such as the upper layers of marine submerged sediments, where the presence of both O_2 and nitrate leads to higher biodegradation rates than when only one TEA is present[253]. The NRB in these environments further degrade products of aerobic hydrocarbon-

degradation metabolism and/or oxidized PHs, such as benzene or hexadecane[74,103,253]. Whether one type of metabolism is predominant or both are equally represented, depends on several factors, such as, concentrations of dissolved oxygen and nitrate, substrate characteristics and composition of bacterial communities[253].

NRB are known to degrade a wide range of aliphatic[248] and aromatic hydrocarbons[249,253,272], preferentially degrading mid-range alkanes (C_{14}-C_{28}) over alkanes with shorter or longer chains[248].

2.5.1.3. Bacteria using other terminal electron acceptors

Other less common bacterial metabolic guilds using other TEAs, such as Fe(III), Mn(IV), methane or organic compounds, are also involved in PHs biodegradation in microaerobic and anaerobic environments. Fe(III) and Mn(IV) are often found in soils and sediments in the forms of crystalline oxides which are usually insoluble or, in the case of Fe(III), can appear in soluble forms chelated with organic ligands, these being particularly found in anaerobic environments[298]. The first bacterium identified as being able to degrade aromatic hydrocarbons in anaerobic conditions was the FeRB *Geobacter metallireducens*[298,299]. Members of *Geobacter* genus are regarded as model organisms of hydrocarbon-degrading FeRB[294,300]. Several uncultivated FeRB, like members of *Chloroflexi* and *Clostridiales,* have been identified in oil contaminated sludge from the petroleum industry[272]. In microcosms of coastal sediments spiked with toluene, the predominant hydrocarbonoclastic FeRB was affiliated to the *Desulfuromonas* genus[263]. FeRB are known to metabolize aromatic hydrocarbons, like BTEX[219,263,301], and in the case of this group of compounds, addition of citric acid has been shown to enhance biodegradation[301]. PH-degrading FeRB have been found in almost undetectable amounts in submerged coastal sediments of an oil contaminated beach[219].

Both iron and manganese reduction can be the predominant metabolisms found in PH-contaminated groundwater. Respectively, *Geobacter* sp. and *Pseudomonas* sp. have been identified as the genera associated with these metabolisms in boreholes. The predominance of these geochemical processes appears to be linked to the presence of high concentrations of soluble iron and manganese found in groundwater. However lower concentrations of nitrate and sulfate reduction have also been detected[226].

While hydrocarbonoclastic Mn(IV)-reducing bacteria have been identified, addition of Mn(IV) in an attempt to stimulate biodegradation of PAHs in mangrove sediments under microaerobic conditions had the opposite effect, causing significant decreases in biodegradation rates, bacterial growth and activity. These results were attributed to the fast conversion of the added Mn(IV) to Mn(II), the latter known to be toxic to microorganisms. Reduction of Mn(IV) to Mn(II)

can happen by microbial and chemical processes, enhanced by the very low redox potential of the sediments[302]. In similar studies involving addition of either $NaHCO_3$, a precursor of CO_2, or Fe(III), none of the TEAs had a significant effect on the biodegradation rates of the PAHs in mangrove sediments[186,188]. A test on the effect of amendment with nitrate, Fe(III) and Mn(IV) showed that only the first had a positive impact in pyrene biodegradation in microaerobic conditions[303]. These results indicate that bacteria able to reduce Fe(III) or Mn(IV) are often less influential in PAHs biodegradation in sediments and soils under microaerobic and anaerobic conditions than metabolic groups such as NRB and SRB.

Most of the organic compounds used by bacteria as TEAs are humic substances. These can be more abundant and active than other TEAs in soils and sediments, and are known to promote pollutant biodegradation, either by serving as electron acceptors or electron shuttles (act as mediator between an electron donor and an electron-accepting compound). So far, only PAHs such as toluene, benzene and phenanthrene, are known to be degraded by bacteria using humic substances as TEAs[304]. There are still very few studies regarding the use of organic compounds as TEAs and even fewer working with isolated and pure cultures. An isolated strain of *Pseudomonas aeruginosa* and other of *Geobacter* sp. were able to degrade phenanthrene and benzene, respectively, using anthraquinone-2,6-dissulfonate, a humic-substance analog, as a TEA[300,305]. *Geobacter* sp. was also able to use Fe(III) as TEA[300]. In the case of *P. aeruginosa* PAH-1, addition of small amounts of fructose enhanced biodegradation under anaerobic conditions[305].

2.5.2. Metabolic pathways of hydrocarbons degradation

Degradation of PHs by microbes involves the oxidation of the hydrocarbons to alcohols, ketones and organic acids. These are often more soluble than the original PHs. Ultimately, all organic compounds, if given the right conditions, are converted to carbon dioxide and water[140]. Small alkanes and cyclic hydrocarbons may be fully oxidized by a single bacterial strain but complete microbial oxidation of more complex hydrocarbons often involves several metabolic pathways found in different strains, thus requiring bacterial consortia[306–308]. There are two main fates for PHs when degraded by the respiration pathway. The first process is oxidative phosphorylation, or simply aerobic respiration, and uses the hydrocarbons as carbon source in the process of energy production. This is the most energetically favorable pathway for energy production. In the second process, bacteria attempt to detoxify their immediate surroundings from PHs by converting them to more soluble products that are then released to the environments[140].

PH degradation occurs within the bacterial cell. Hence, the process is limited by cellular uptake[309]. While some PHs can be dissolved in the aqueous phase, others are very hydrophobic and

insoluble in water, uptake of these compounds occurs either at the water-oil interface or is enhanced by surfactant micelles[265,309,310]. Trans-membrane transport of hydrocarbon molecules that come in direct contact with the cell involves passive diffusion or active transport through membrane proteins[309,311]. However, there is still very little information regarding the specific mechanisms of hydrocarbon uptake by bacteria, especially considering that different bacteria may operate different mechanisms[265,309].

Catabolic metabolism of aliphatic, cyclic and aromatic PHs starts with a common initial step in which the substrate is activated. In aerobic conditions, hydroxylation reactions using O_2 as a reactant are responsible for hydrocarbons activation[267]. This is performed by a diverse group of enzymes called oxygenases, specifically monooxygenases and dioxygenases, which introduce one or two hydroxyl groups in the hydrocarbon chain, respectively (Figure 2.4, A and B). The oxygen atom of the hydroxyl groups originates from the cleavage of O_2, while the remaining atom is reduced to water[312]. This step transforms hydrocarbon molecules with low chemical reactivity into reactive oxygen species[265]. In anaerobic conditions other non-O_2 depending processes, such as methylation and carboxylation, are used. Generically, initial activation is followed by other oxidative processes that lead to the formation of carboxylic acids, which through subsequent steps of the peripheral degradative pathways, are converted into central precursor metabolites, such as acetyl-CoA, succinate, and pyruvate. These are used in central metabolic pathways, such as the tricarboxylic acid cycle, and converted into biomass[195 267]. These pathways will be further discussed in the following sub-sections. The rates at which hydrocarbons are degraded by bacteria, both under aerobic and anaerobic conditions, depend on the bioavailability of the compounds and on the metabolic pathways available in the cell[140]. However, the energy yield produced by anaerobic degradation of hydrocarbons is lower than that produced under aerobic conditions. As such, the anaerobic process is only energetically competitive in environments with low or null oxygen concentrations[140].

Key genes associated with PHs degradation and widely distributed in hydrocarbon-degrading bacteria, such as those codifying for mono- and dioxygenases, have been used as molecular markers indicative of the ability of the community to perform hydrocarbon biodegradation[313–316]. The diversity of genes and homology of DNA fragments associated with hydrocarbon degradation and the bacterial hosts where they are found, suggest that these genes can be transferred horizontally and suffer gene fusion and duplication thus increasing bacterial adaptability to different substrates[314]. Genes encoding for hydrocarbon-degradation pathways have been found in plasmids, and encased between transposons and transposon-like sequences, which are very mobile DNA molecules and sections, respectively[192,317,318]. It is possible for these plasmids to be horizontally transferred into new bacterial strains, thus increasing the degradative potential of other members of the community upon selective pressure[192]. In many cases, genes encoding proteins involved throughout hydrocarbon

degradation are arranged in operons[319]. While enzymes involved in the initial activation of hydrocarbon molecules are often specific for certain types or groups of hydrocarbons, many of the other enzymes involved in hydrocarbon degradation have a broader range of substrates[319]. Furthermore, bacterial strains may have more than one set of enzymes equipped to deal with hydrocarbon-degradation. This increases the amount of substrates that can be catabolized and can confer a competitive advantage to the strain, while increasing the plasticity of hydrocarbonoclastic populations[267,315].

The best studied genes associated with hydrocarbon degradation are those involved in the initial oxidation. In general, these have been found to be tightly regulated by a specific regulator that only allows for gene expression when the adequate hydrocarbons are present. Additionally, other mechanisms associated with cell responses to environmental conditions can influence the expression of hydrocarbon degradation genes[265]. Some examples include product repression, in which excessive amounts of degradation products inhibit the activation of the genes associated with hydrocarbon-degradation[320], or catabolite repression control, where the presence of more easily degraded substrates which are preferred, inhibits the expression of genes associated to degradation of more complex carbon sources, such as hydrocarbons[321,322]. Understanding these mechanisms can be fundamental to anticipate the degree of success of particular bioremediation strategies and improve them.

The knowledge obtained by the study of metabolic pathways of hydrocarbon-degrading bacteria can have several practical applications[257]. It makes it possible to identify specific nutritional requirements to enhance biostimulation strategies, to infer on metabolic pathways operating at contaminated sites by identification and quantification of specific metabolites, and to detect the presence of metabolic pathways responsible for the production of toxic intermediaries. However, due to the diversity of hydrocarbon degradation pathways, much is yet unknown and many known pathways still suffer from large knowledge gaps. The advent of molecular techniques has been providing clues to missing links between pathways. Genetic and metabolite profiling are tools used in identification and assessment of the activity of metabolic pathways involved in *in situ* degradation of hydrocarbons[313,323,324].

2.5.2.1. Aliphatic hydrocarbons

In alkane degradation, initial hydroxylation is performed by a multimeric monooxygenase or a cytochrome P450 monooxygenase, resulting in the formation of alcohols[267]. The former is the most common process and the latter is more characteristic of bacteria containing CYP153 enzymes[325,326]. Generally, hydroxylation occurs at a terminal methyl group and the alcohol formed is the alkane

correspondent alkan-1-ol[327]. However, subterminal oxidation has been observed in some instances[328]. The primary alcohol is then converted by subsequent oxidations into an aldehyde and latter a fatty acid or, in some cases, a dicarboxylic acid, which follows to the beta-oxidation (also known as fatty acid) pathway generating acetyl-CoA[267]. In cases of initial subterminal oxidation, the secondary alcohol is converted into a ketone and then oxidized into an ester, which in turn is hydrolyzed forming an alcohol and a fatty acid[265]. The multimeric monooxygenase comprises a particulate membrane-bound hydroxylase, a rubredoxin, and a rubredoxin reductase. A model enzyme of particulate membrane-bound hydroxylase is AlkB, found in *Pseudomonas putida* GPo1. Many Proteobacteria and Actinobacteria members contain genes similar to *alkB*, which codify for structurally similar enzymes but with different specificity and substrate range, which usually varies between C_5 to C_{11} *n*-alkanes[265,326]. *alkB*-like genes represent some the most used molecular markers in detection and quantification of alkane degrading bacteria and communities degradative potential[313,315]. Operons *alkBFGHJKL*, of which the *alkB* gene is part of, and *alkST,* codify for the necessary enzymes to convert *n*-alkanes into fatty acids[329]. Bacteria may codify for more than one particulate membrane-bound hydroxylase that may be expressed under different conditions or used for different substrates due to differential regulation[265]. Since most catabolic enzymes used in the bacterial metabolisms of aliphatic hydrocarbons are not specific for a single compound but interact with a family of similar structural molecules[325], most bacteria are able to degrade more than one specific hydrocarbon[330]. Interestingly, many bacteria not usually considered to be hydrocarbonoclastic, present homologous genes to alkane-hydroxylases genes, that in some cases codify for functional alkane hydroxylases[331].

Alkenes are naturally more chemically reactive than alkanes and aromatic hydrocarbons due to the presence of double bonds thus making them more easily degraded[332]. Similar to alkanes, the first step in their degradation involves monooxygenases which insert an oxygen atom in the double bond site thus converting alkenes into epoxide molecules[333]. However epoxides are known to be toxic to monooxygenases and other cell components[334]. As such bacteria using this mechanism are usually also equipped to neutralize the epoxides. Depending on the bacteria, this can be achieved through different pathways, by using enzymes such as oxide hydrolases, glutathione-S-transferases, carboxylases or isomerases. The resulting products, which can be keto acids, ketones, aldehydes or diols, are then further degraded similarly to the alkanes degradation pathways[327].

Branched alkanes are usually more recalcitrant than linear alkanes and alkenes[335]. Bacterial biodegradation starts by activation of the *iso*-alkanes through a hydroxylation mechanism that converts them into acids or ketones which then form the same pathways as described for alkanes[327,336,337].

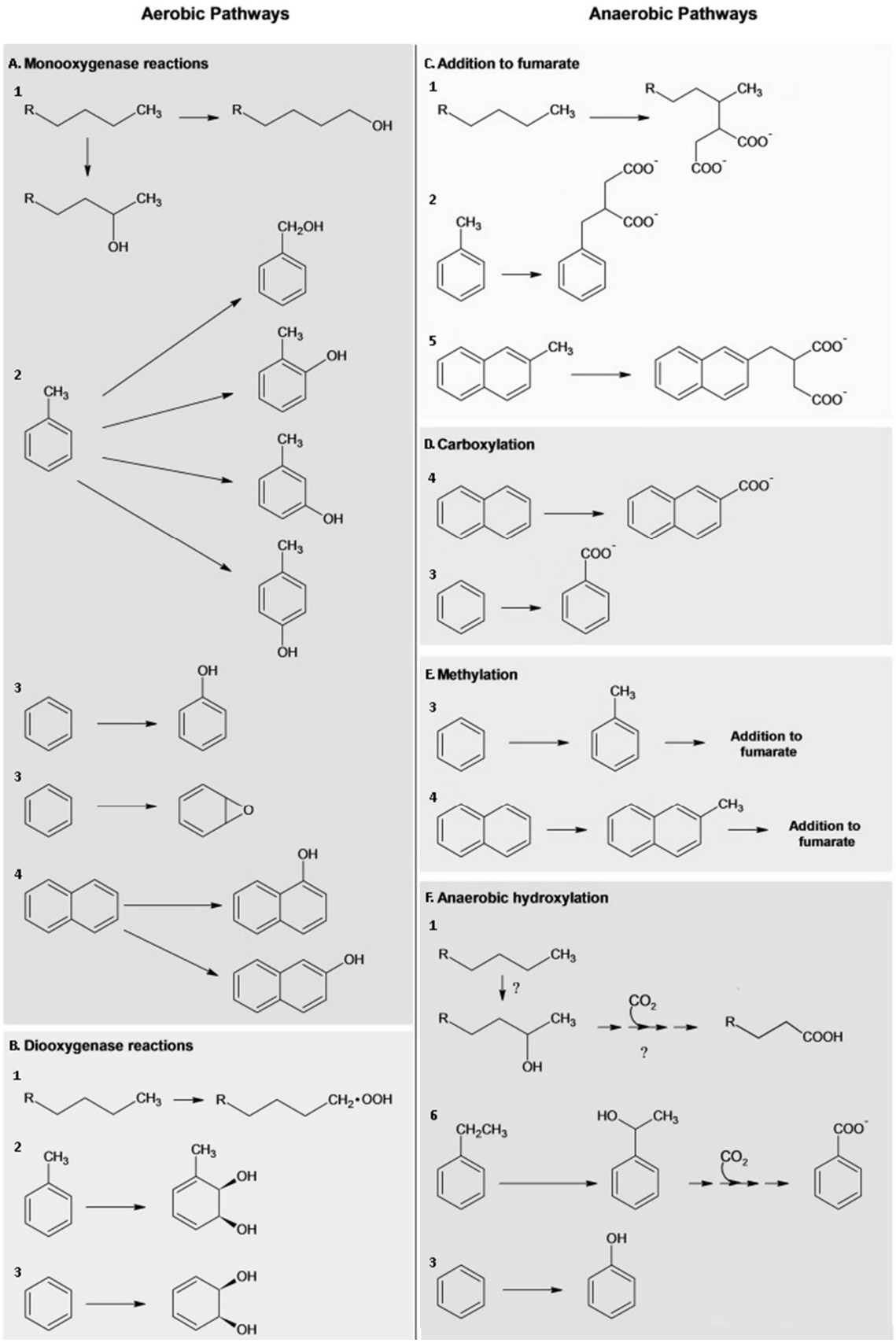

Figure 2.4 – Examples of initial activation pathways of hydrocarbons under aerobic and anaerobic conditions. 1, *n*-alkane; 2, toluene; 3, benzene; 4, naphthalene; 5, 2-methylnaphthalene; and 6, ethylbenzene[225].

Cycloalkanes are even more resistant to bacterial biodegradation due to their toxicity and low solubility. In some cases they are degraded by several strains performing cometabolism[306,307]. Similar to the alkane degradative pathways, the first step involves monooxygenases that transform the cycloalkanes into the corresponding alcohols or ketones[327,338]. These are either used as substrate by other organisms or follow an oxidative pathway which leads to their conversion into dicarboxylic acids, particularly adipic acids, which are subjected to beta-oxidation[327,339].

Due to the lack of O_2 available in anaerobic conditions, the activation of the hydrocarbon molecule occurs by non-O_2 dependent mechanisms, such as carboxylation, fumarate addition, hydroxylation of double and triple bonds (Figure 2.4: D, C and F, respectively) and reverse methanogenesis[267,327]. Carboxylation occurs at the C-3 atom and a C_2 molecule is lost, thus creating a carboxylic acid[340]. In fumarate addition, fumarate is introduced in the alkane chain at the subterminal or terminal carbon by alkylsuccinate synthase (Ass) which leads to the formation of (1-methyl- alkyl)succinates or 2-alkylsuccinates. These are latter transformed into fatty acids through still unclear steps involving carbon skeleton rearrangement, decarboxylation and finally beta-oxidation before entering central metabolic pathways[324,341]. Genes *assA/masD* and *bssA,* which encode for enzymes of fumarate addition pathways and the products of hydrocarbon activation step, (1-methyl- alkyl)succinates, are commonly used as molecular and biochemical markers, respectively, for detection of this type of hydrocarbon activation[324]. This mechanism is likely also used in the initial activation of cycloalkanes[342]. Several possible pathways have also been proposed for initial oxidation of methane under anaerobic conditions by SRB, FeRB and MnRB, especially reverse methanogenesis. However contradictory results make further investigation necessary[291]. The main product obtained through this pathway is acetyl-CoA which is complexly oxidized to CO_2 by central metabolic pathways. The most common of which in SRB being the Wood-Ljungdahl pathway[290].

Anaerobic degradation of alkenes has been found mainly in SRB[343], in which initial oxidation is performed either by addition of carbon units to subterminals[344] or hydration of the double bond[345].

2.5.2.2. Aromatic hydrocarbons

As previously mentioned, aromatic compounds are chemically stable and hydrophobic. As such, before ring cleavage, bacteria must destabilize the benzene ring by performing chemical modifications. This is achieved by oxidation of an aromatic ring by monooxygenases or dioxygenases[257]. Monooxygenases catalyze the successive addition of hydroxyl groups to the aromatic ring, resulting in the formation of phenols and latter catechols (Figure 2.4, A)[346]. Dioxygenases promote reductive dihydroxylation of the aromatic ring obtaining cis-dihydrodiols as products, which are then converted to catechols by cis-dihydrodiol dehydrogenases (Figure 2.4, B)[347].

The central intermediaries formed in the peripheral pathways can be catechols, which have cis-dihydrodiols groups[348], or hydroxy-substituted aromatic carboxylic acids[349].

Central pathways involve the de-aromatization of the central intermediates, followed by ring cleavage by dioxygenases leading to the formation of carbon dioxide and tricarboxylic acids[257]. These products can be then used for the formation of biomass.

Anaerobic degradation of aromatic hydrocarbons is metabolically more diverse than aerobic degradation. The first step in anaerobic peripheral pathways involves the reduction of the aromatic hydrocarbons promoted by synthases, dehydrogenases and carboxylases, which will result in the activation of the aromatic ring[257]. So far there are five known pathways by which this can be achieved: fumarate insertion[350], carboxylation[351], methylation[352], O_2-independent hydroxylation[353] (Figure 2.4: C, D, E and F, respectively) and phosphorylation[354]. Due to the diversity in possible pathways, a diverse group of metabolites can be obtained as central intermediates, with the most common being benzoyl-CoA and its derivatives. Like in aerobic metabolism, central pathways consist in de-aromatization and cleavage of the aromatic ring. The pathway followed depends on the central intermediate available and have as final products methane, carbon dioxide and tricarboxylic acids[257].

2.6. Conclusions

Hydrocarbons biodegradation by bacteria is an important biological process ubiquitous in environments containing petroleum. It is responsible for changes to crude oil composition in oil reservoirs or removal of hydrocarbons in contaminated environments. The bacteria directly involved in this process are called hydrocarbonoclastic and are of particular interest for application in MEOR and bioremediation strategies. However, most of the current knowledge concerning PHs biodegradation, hydrocarbonoclastic bacteria and the metabolic pathways involved in PHs biodegradation are essentially restricted to aerobic conditions. In the last years some progress has been made regarding oxygen-limiting conditions, but much is still to be understood. Marine environments containing PHs, either due to natural seepage or contamination, are prime environmental matrixes for further studies regarding anaerobic PHs biodegradation. The focus of future research needs to be set on the assessment of the bacterial communities present in these environments that are directly or indirectly involved in PHs biodegradation and on the isolation of facultative anaerobic hydrocarbonoclastic bacteria with biotechnological potential for application in anaerobic MEOR and bioremediation strategies.

2.7. Acknowledgments

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Chapter 3

CHAPTER 3

Characterization of anaerobic hydrocarbonoclastic bacterial communities from subsurface sediments of active and inactive deep-sea mud-volcanoes

Patrícia M. Domingues, Francisco J. R. C. Coelho, Mário Simões, Adelaide Almeida, Luísa S. Serafim, Newton C. M. Gomes, Ângela Cunha

3.1. Abstract

Deep-sea sediments from mud volcanoes (MVs) are often exposed to natural hydrocarbon seepage. This work aimed at understanding how the bacterial communities of active and inactive MVs respond to the presence of crude oil under oxygen-limiting conditions. Selective cultures with or without crude oil and/or nitrate, as an alternative electron acceptor, were inoculated with sediments from MVs and incubated for a total of 15 weeks under anaerobic conditions. After incubation, the structure of bacterial communities in enrichment cultures was assessed by 16S rRNA gene pyrosequencing. Active and inactive MV sediments originated distinct bacterial communities independently of amendments, with Alphaproteobacteria and Bacilli dominating in the selective cultures of active and inactive MVs, respectively. The presence of nitrate and/or crude positively affected the Firmicutes in the active MV cultures. In particular, Planococcaceae were stimulated by the presence of crude oil but negatively affected by nitrate amendment. Crude oil positively affected the relative abundance of Erythrobacteraceae and negatively affected Actinobacteria, while amendment with nitrate tended to favor Nocardioidaceae. In cultures inoculated with inactive MV sediment, the presence of nitrate or crude oil led to an increase of Bacillaceae in detriment of other Bacillales. In these cultures, crude oil also caused an increase of the relative abundance of Acidimicrobiales and Flavobacteriaceae. GC-MS was used to analyze the biodegradation of *n*-alkanes, monoaromatic and polycyclic aromatic hydrocarbons in all enrichment cultures. Overall, bacterial communities from active MVs were able to degrade *n*-alkanes $<C_{13}$ and monoaromatic hydrocarbons, while those from inactive MV were able to degrade a wider range of polycyclic aromatic hydrocarbons. Nitrate induced the communities from active MV to degrade medium *n*-alkanes (C_{10}-C_{13}) instead of smaller molecules ($<C_{10}$). These results can help to identify bacterial groups present in these particular ecosystems that are involved in hydrocarbon biodegradation, as well as help predict how the bacterial communities of these MVs respond to crude oil contamination.

3.2. Key words

Bacterial diversity; deep-sea sediments; advanced sequencing; anoxia; nitrate reduction.

3.3. Introduction

The deep-sea is one of the Earth's largest ecosystems, comprising around 60% of its solid surface[355]. Overall, it is considered to be an extreme environment due to lack of light, slow currents, scarce sediment accumulation and low supply of organic matter, low temperature (< 5°C), high pressure (> 10 MPa) and high salinity (34.3-35.1 g/L)[356,357]. Exceptions include hydrothermal vents and cold seeps, where petroleum hydrocarbons (PHs) and other components are expelled to the deep-sea floor from within deep sediment layers. In these habitats, metabolic activity and biomass are higher[250,358].

Submarine or off-shore mud volcanos (MVs) are a type of cold seeps characteristically elevated from the seafloor with a diameter of up to 10 km and generally displaying a peak, flat surface or crater at the top[359]. They are found mainly along continental margins, resulting from the over-pressuring of sediments likely by compression forces of tectonic plates or gravitational forces due to PH reservoirs activity[250,360–362]. This leads to the intrusion and seepage of pressurized fluids, forming muds, which can be composed by gas (mainly methane), water, liquid PHs and solid particles[363,364].

Both acute and chronic releases of PHs lead to environmental damage, which can present negative effects from short- to long-term[142,144,150,160]. Natural sources are the predominant single source of PHs input in marine environments[140,157]. PHs from natural sources reach the deep-sea mainly from seeps, such as MVs. PHs of anthropogenic origin can also have a strong impact in these environments and reach the deep-sea by lateral advection, diffusion and sinking[140,231]. Surface deep-sea sediments are known sinks for PHs, especially in deep basins and canyons and in particular polycyclic aromatic hydrocarbons (PAHs)[231,365]. Recalcitrance of PHs in sediments increases with depth, with the most labile PHs being degraded in the aerobic layers[273]. In general, in seep sediments, the aerobic zone is limited to the few uppermost millimeters, while in adjacent zones, oxygen is available in the sediment column up to tens of centimeters[366]. Due to their low volatility, solubility and high hydrophobicity, aromatic hydrocarbons often adsorb to suspended particles that accumulate in the bottom sediments[151,184]. Hydrocarbons sorption to sediment particles is positively affected by increasing salinity, due to salting out effects[367] thus enhancing PH accumulation in sediments of hypersaline environments, such as the seafloor.

Natural anaerobic biodegradation of PHs is known to occur in the deep ocean near natural seeps as long as the necessary terminal electron acceptors (TEAs) are present[279,280,368]. The most used TEAs by MVs microbial communities are oxygen and sulfate, since they are the most abundant, but nitrate, Fe(III) and Mn(IV) may also be used if available[361]. However, sulfate-reducing bacteria (SRB) are known to be implicated in souring of crude oil[128] and corrosion of infrastructures[286], making them undesirable in some biotechnological applications, such as MEOR. While nitrate-

reducing bacteria (NRB) are also known to be able to degrade a wide range of PHs[242,248] without causing, and in fact diminishing souring of crude or corrosion, by inhibiting SRB growth[297]. Nitrate is one of the most thermodynamically favorable electron acceptors used under anaerobic conditions[35]. Facultative anaerobic hydrocarbon-degrading bacteria are ubiquitous in anaerobic environments, such as deep-sea water[282]. Since crude oil seepage is usually a slow process, in areas affected by natural seeps organisms have adapted to the presence of PHs either by developing tolerance or even by taking advantage of hydrocarbons by using them as carbon source[158,159]. Furthermore contaminated areas are often characterized by lower bacterial diversity than uncontaminated ones[159]. Extensive bacterial degradation of PHs is usually performed by bacterial consortia, with different bacteria being involved in several progressive steps of the biodegradation process. Some bacteria directly degrade the PHs while others degrade metabolites of primary degraders[242,308]. In general, hydrocarbonoclastic bacteria are able to degrade more than one compound but often particular bacterial groups are specialized in some chemical families[223,269].

Despite its extension, the deep-sea is one of the least studied marine habitats[231,356]. Many new microorganisms are being discovered and the deep-sea is often regarded as a hidden reservoir of bacterial diversity, novel metabolic activity and enzymes with industrial and environmental interest[369]. In general, bacterial diversity in the deep-sea is high and communities are functionally versatile and highly adaptable to alterations in organic matter inputs[356]. As such, this study aims to expand the knowledge of deep-sea MVs bacterial communities. It aims particularly at identifying key players in natural hydrocarbon biodegradation in active and inactive MVs ecosystems, since both ecosystems are naturally subjected to different inputs of PHs, the first being mainly affected by natural seepage and the later by PHs of anthropogenic sources. The structure of bacterial communities was assessed as well as the responses of bacterial communities from active and inactive MVs sediments to the enrichment of crude oil as sole carbon source and the presence or absence as nitrate as TEA.

3.4. Materials and Methods

3.4.1. Study area and sampling

The surface of Gulf of Cadiz deep-sea represents a complex geological system. The convergence of the African plate towards the Euroasian plate and the destabilization of gas-hydrate rich sediments lead to extensive mud volcanism in the accretionary wedge that crosses the Gulf of Cadiz (AWGC), at depths between 200 m and 5000 m (Figure 3.5). This is particularly evident along transform fault zones, such as the strike-slip faults in the South-West Iberian Margin[370,371]. Here, natural seepage of gas and hydrocarbons, particularly methane, sustains an ecosystem based on

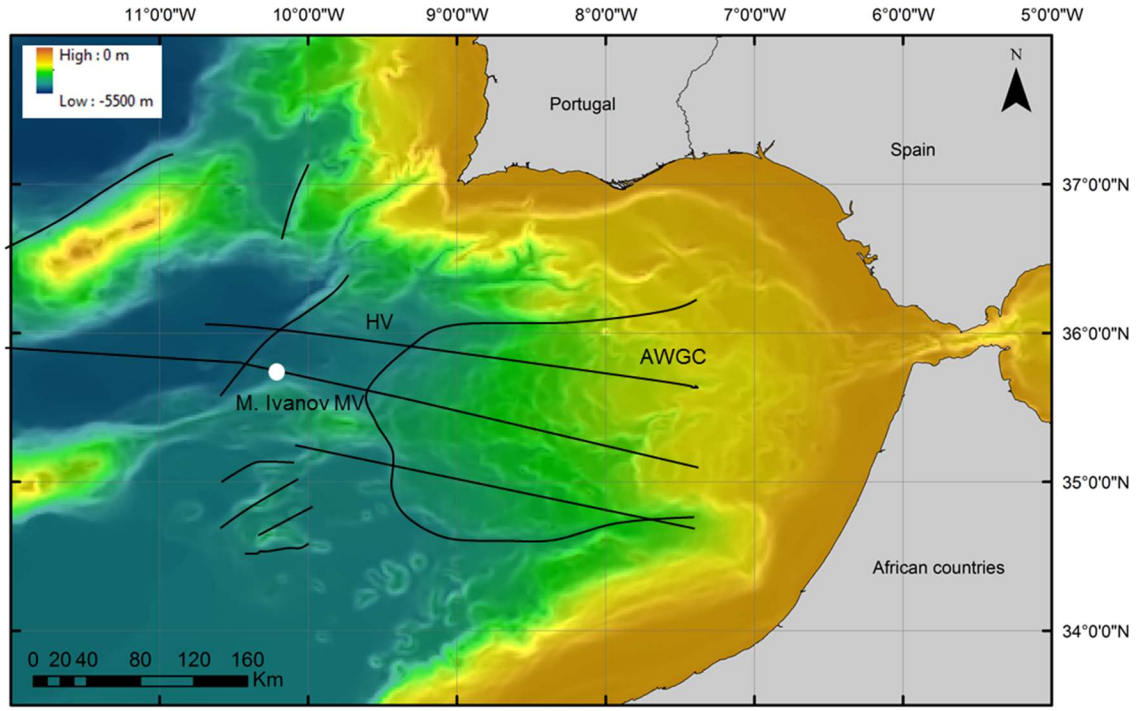

Figure 3.5 – Location of the sampling site, Mikahail Ivanov MV. HV: Horse shoe valley; AWGC: accretionary wedge that crosses the Gulf of Cadiz. The maps were generated using ArcGIS 10.0 (version 10.0; http://www.esri.com/) software with bathymetry data previously published [526].

chemosynthetic metabolism[368]. Furthermore, in the Gulf of Cadiz, MVs occur in areas of intense maritime traffic, hence subjected to anthropogenic inputs of hydrocarbons and highly exposed to the risks related with accidental spills and shipping accidents[372].

The samples used in this study were collected during the SWIMGLO/Transflux M86/5 cruise onboard the RV Meteor[373] at the Mikahail Ivanov MV located on the AWGC and formed by several craters. Samples were collected at northwest crater, station 348, which presents no indication of seepage activity, and at the active southeast crater, station 329 (Table 3.5). Sampling was conducted with a box corer. Sub-samples of 0.5-1g of subsurface sediments (10 cm below seafloor) were collected, immediately deep-frozen (-80 °C), kept on dry ice during transportation, and stored at -80 °C until further analysis.

Table 3.5 – Description of sampling stations at the Mikahail Ivanov MV

Station	Date (dd.mm.yy)	Latitude (D:M)	Longitude (W)	Depth (m)	Activity
329	01.03.12	35:44.34	10:12.06	4492	Active
348	05.03.12	35:44.41	10:12.18	4497	Inactive

3.4.2. Anaerobic enrichment cultures

For the preparation of anaerobic enrichment cultures of hydrocarbonoclastic and/or NRB from deep-sea MVs sediments, full marine medium for NRB was used, as described in Widdel (2010), with some adaptations[98]. Resazurin 0.001 g/L was added to the main solution, the vitamin mixture and thiamine solution were replaced by RPMI 1640 Vitamins Solution 100X (Sigma) 10 mL/L, KNO_3 0.01 M was used instead of $NaNO_3$ and cycloheximide 0.01 g/L was added to the final medium. Removal of oxygen from the media was conducted as described in Plugge (2005) and Wolfe (2011)[97,374].

With the aim of assessing the effects of nitrate and crude oil amendment on bacterial communities of active and inactive MVs, a complex experimental design was prepared involving 28 serum vials, including three cultures per condition prepared with different sediment sub-samples from the same corer (henceforth identified as a, b and c; Table 3.6). Abiotic controls for all conditions were also prepared. Enrichment cultures were prepared in 50 ml serum vials sealed with butyl rubber stops and aluminum crimps. Serum vials were filled up to 9:10 capacity, corresponding to a final volume of 45 mL, with medium prepared according to the enrichment factors. In cultures without nitrate as TEA, KNO_3 was absent (0 and C). While in cultures with light Arabian crude oil, 1% (v/v) of the carbon source was added (C and NC). Small aliquots of crude oil were sterilized by exposure to UV radiation for 4 hours and added to the medium in the sealed serum vials before autoclaving. For the initial cultures, 50 mg of sediment was added to 45 mL culture medium and in the two subsequent transfers to fresh medium, 10% (v/v) of the previous culture was used as inoculum. As a final step, after inoculum addition and sealing of the vials, the headspace of the enrichment cultures was filled with N_2. Vials were kept in inverted position during the incubation in the dark[98] at 25 °C. After each re-inoculation in fresh medium, the cultures were incubated for 5 weeks, totaling 15 weeks of incubation.

After incubation, 10 mL aliquots of each enrichment culture were centrifuged at 4696 x g for 45 min. The pellet was stored at -20 °C in a microtube and covered with absolute ethanol for later

Table 3.6 – Identification of the enrichment cultures of deep-sea sediments in relation to inoculum and enrichment factors for anaerobic metabolism.

		Enrichment factor			
		No amendment	*NO_3^-*	*Crude oil*	*NO_3^- + Crude oil*
Inoculum	Abiotic control	Ø-0	Ø-N	Ø-C	Ø-NC
	Active MV	A-0	A-N	A-C	A-NC
	Inactive MV	I-0	I-N	I-C	I-NC

DNA extraction. The supernatant was stored at -20 °C and later used in chemical oxygen demand (COD) determination and high-pressure liquid chromatography (HPLC) analysis. The remaining volume of liquid cultures was stored at -80 °C in the sealed vials.

3.4.3. Molecular analysis of bacterial communities from enrichment cultures

3.4.3.1. DNA extraction of selective cultures and PCR-amplification of 16S rRNA gene fragments

After the final incubation period, DNA was extracted from 10 mL aliquots of each enrichment culture using the FastDNA Spin kit for soil (MPBiomedicals, USA). DNA extracts were stored at -20 °C.

Initial PCR-amplification of the 16S rRNA gene fragments of the bacterial communities was performed using the primers U27F and 1492R[375]. The composition of the reaction mixture (25 µL) was 1 µL of sample, 12.5 µL DreamTaq™ PCR Master Mix (Thermo Fisher Scientific), 0.25 µL of each primer, 1 µL BSA (2 g/L; Sigma), and 10 µL dH_2O. The PCR cycle was as described in Domingues *et al.* (2013)[205]. The PCR products were then submitted to nested PCR, using the Bacteria domain specific primers 984F-GC and 1378R[376]. The reaction mixtures had a total of 25mL and contained 1 µL sample, 12.5 µL DreamTaq™ PCR Master Mix, 0.25 µL of each primer, 0.5 µL acetamide (Fluka), and 10.5 µL dH_2O. The second PCR conditions were those as described in Oliveira *et al.* (2014)[145].

3.4.3.2. Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed with the DCode System (BioRad): 7 µL of PCR products containing approximately equal amounts of DNA were loaded onto 6–10% (w/v) polyacrylamide gel, with a denaturing gradient of 40–58%, in 1 × TAE buffer. Electrophoresis was performed for 16 h at 70 V at 60°C in 1 × TAE buffer. The gels were silver-stained following the protocol described in Heuer *et al.* (2001)[377]. The profiles were analyzed with GelCompar 4.0 software (Applied Maths, Belgium) and cluster analysis was performed with PRIMER v5 software (Primer-e, UK)[205].

3.4.3.3. Sequencing

Bacterial communities of the deep-sea enrichment cultures were characterized by a 16S rRNA based barcoded pyrosequencing approach. The V3-V4 region of the bacterial 16S rRNA gene was amplified using barcoded fusion primer V3 Forward (5′ -ACTCCTACGGGAGGCAG-3') and V4 Reverse (5′ -TACNVRRGTHTCTAATYC-3') with the Roche 454 titanium sequencing adapters[378]. GS 454 FLX Titanium chemistry was used to sequence equimolar concentrations of the PCR products, according to the instructions of the manufacturer (Roche, 454 Life Sciences,

Brandford, CT, USA). Sequences have been uploaded to the NCBI Short Read Archive, and are associated to the BioProject PRJNA394655.

Sequencing analysis was performed using QIIME software (Quantitative Insights into Microbial Ecology – http://www.qiime.org/; version 1.8.0)[379] following previously described methods[380,381]. In QIIME, fasta and qual files were used as input for the split_libraries.py script. Default arguments were used except for the minimum sequence length, which was set at 218 bps after removal of forward primers and barcodes; reverse primers were removed using the 'truncate only' argument and a sliding window test of quality scores was enabled with a value of 50 as suggested in the QIIME description for the script. In addition to user-defined cutoffs, the split_libraries.py script performs several quality filtering steps (http://qiime.org/scripts/split_libraries.html). Operational taxonomic units (OTUs) were selected using UPARSE with usearch7[382]. The UPARSE sequence analysis tool provides clustering, chimera checking and quality filtering on de-multiplexed sequences. Chimera checking was performed using the UCHIME algorithm. First reads were filtered with the –fastq_filter command and the following arguments –fastq_trunclen 250 –fastq_maxee 0.5 –fastq_truncqual 15. Sequences were then dereplicated and sorted using the –derep_fulllength and –sortbysize commands. OTU clustering was performed using the –cluster_otus command. An additional chimera check was subsequently applied using the –uchime_ref command with the gold.fa database (http://drive5.com/uchime/gold.fa). AWK scripts were then used to convert the OTUs to QIIME format. In QIIME, representative sequences were selected using the pick_rep_set.py script by the 'most_abundant' method. Reference sequences of OTUs were assigned taxonomies using default arguments in the assign_taxonomy.py script in QIIME with the rdp method (SI Table 3.1)[383]. In the assign_taxonomy.py function, a fasta file containing reference sequences from the gg_13_8 greengenes database release and the rdp classifier method (ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8_otus.tar.gz) were used. Finally, the make_otu_table.py script in QIIME was used to generate a square matrix of OTUs x samples.

Variation in composition among sites was assessed using principal coordinates analysis (PCO). The PCO was generated using the cmdscale() function in the R base package and wascores() function in vegan. Prior to the PCO, the raw data was log (x+ 1) transformed and used to produce a distance matrix with the Bray-Curtis index with the vegdist() function in vegan30. The R vegan adonis() function for permutational multivariate analysis of variance (PERMANOVA) was used to test for significant variations in composition between sediments. In the adonis analysis, the Bray-Curtis distance matrix of OTU composition was the response variable with samples as independent variables. The number of permutations was set at 999.

A binary (1/0) matrix was constructed taking into account the presence or absence of individual OTUs. Cluster analysis was performed using the PRIMER v5 software (Primer-e). The binary matrix was transformed into a similarity matrix using the Bray-Curtis measure. In order to compare the diversity between the different enrichment microcosms, the Shannon–Weaver diversity index (H') was calculated[384].

3.4.4. Chemical oxygen demand

Soluble COD was measured using the COD Cell Test Method: photometric 10 – 150 mg/l Spectroquant® (Merck) and the Spectroquant® Picco COD/CSB colorimeter (Merck). One mL of supernatant of each enrichment culture was diluted 1:20 with dH_2O to keep the Cl^- levels below 2000 mg/L, as confirmed by the MquantTM Chloride Test (Merck). Results presented correspond to the average of all three enrichment cultures under the same conditions with different sub-samples.

3.4.5. Production of organic acids

Acetic, propionic, butyric and valeric acids were measured by HPLC. For this, 600 µL of each supernatant sample mixed with H_2SO_4 0.0035 M and lactic acid 0.46 g/L (as internal standard) was filtered using centrifuge tube filters with a cellulose acetate membrane, 0.2 µm pore size at 8000 rpm for 10 min. Samples were then injected (Auto-sampler HITACHI L-2200, Hitachi, Ltd., Chiyoda, Japan) in an ion-exclusion column Rezex ROA-Organic Acid H+ 8% (Phenomenex, USA) at 65 °C (Oven Gecko-2000, CIL Cluzeau, France), and analyzed by a refractive index detector (HITACHI L-2490, Hitachi, Ltd., Japan). The eluent 0.005 N H_2SO_4 was pumped at a flow rate of 0.65 mL/min (HITACHI L-2130 pump, Hitachi, Ltd., Japan) at room temperature. The eluent was prepared with milli-Q water and filtered with a cellulose acetate membrane, 0.22 µm pore size. The concentrations of organic acids in g/L were determined by application of calibration curves of each analyzed compound obtained using standards of known concentrations, between 0.0078 to 1.00 g/L for acetate and 0.0039 to 0.50 g/L for propionate, butyrate and valerate.

3.4.6. Biodegradation of petroleum hydrocarbons

Gas chromatography coupled with mass spectrometry (GC-MS) was used to quantify the variation of PHs in the different enrichment cultures. The organic phase of each enrichment culture was removed from the cultures frozen at -80 °C and stored at -20 °C shortly before GC-MS analysis. Prior to injection, 70.0 µL of each sample, was mixed with 282.0 µL of 1,2,4-triclorobenzene (4 g/L) as internal standard for the alkanes run, 112.8 µL hexamethylbenzene (0.1 g/L) as internal standard for the PAHs run and 1035.2 µL of dichloromethane. GC-MS analysis was performed in a GC-2010 Plus gas chromatograph (Shimadzu), equipped with an automatic injector AOC-20i (Shimadzu) and coupled with a GCMS-QP2010 Ultra mass spectrometer (Shimadzu), using a Chrompack CP-Sil 8 CB low bleed/MS capillary column (30m length, 0.25mm ID and 0.25 µm film thickness). Helium

was used as the carrier gas at a constant linear velocity of 40 cm/s. Injector temperature was 320 °C. For quantification of alkanes (C_7 to C_{32}) and monoaromatic hydrocarbons (MAHs: ethylbenzene, toluene and *o*-, *p*- and *m*-xylenes) a split ratio of 50 was used. The oven temperature was set at 50 °C during 5 min, raised to 200 °C at a rate of 5 °C/min, then to 300 °C at a rate of 10 °C/min and at last kept at 300 °C during 15 min. For the analysis of the 16 PAHs in the EPA watch list[385] the splitless injection mode was used with a sampling time of 5 min. The oven temperature was set at 60 °C during 1 min, raised to 200 °C at a rate of 10 °C/min and held for 2 min, raised again to 300 °C at a rate of 5 °C/min and at last kept at 300 °C during 8 min.

The MS interface temperature was kept at 320 °C and the MS ion source temperature at 250 °C. For alkanes and MAHs, the MS was operated in the scan mode at 70 eV using a range of m/z 30–1000 and the detector voltage was 0.1 kV. Solvent cut time was at 2 min. For PAHs the mass spectrometer was operated in SIM mode at 70 eV using the ranges detailed below and the detector voltage was 0.1 kV. Solvent cut time was at 7.1 min. The 16 PAHs and the internal standard were detected in the following time windows: m/z 128 (naphthalene) from 7.80–8.20 min, m/z 147 (1,2,4-trichlorobenzene) and m/z 152 (acenaphthylene) from 11.35–11.90 min, m/z 154 (acenaphthene) from 12.00–12.30 min, m/z 166 (flourene) from 13.25–13.55 min, m/z 178 (phenanthrene and anthracene) from 15.55–16.20 min, m/z 202 from 19.80–20.30 min (fluoranthene) and from 20.75–21.20 min (pyrene), m/z 228 (benz[*a*]anthracene and chrysene) from 26.15–26.90 min, m/z 252 from 30.80–31.60 min (benzo[*b*]flouranthene and benzo[*k*]flouranthene) and from 32.05–32.60 min (benzo[*a*]pyrene), m/z 276 (indeno[1,2,3-*cd*]pyrene) and m/z 278 (dibenz[*a,h*]anthracene) from 36.15–36.90 min, and m/z 276 (benzo[*g,h,i*]perylene) from 37.00–45.00 min.

Chromatograms were automatically processed using the software LabSolutions GCMS v4.20 (Shimadzu). Manual adjustments were made whenever necessary. For identification purposes, the retention times (in SIM and scan modes) and mass spectrum (only in scan mode) of the analytes were compared with standards. Identification was also assisted by comparing the mass spectrum of each peak with information available from mass spectral libraries (Wiley 229 and US National Institute of Science and Technology (NIST) v. 2.2).

The degradation or formation of PHs was assessed by comparing the composition of the hydrocarbons recovered from the enrichment cultures inoculated with sediments with those from abiotic controls. The pairs benz[*a*]anthracene and chrysene, benzo[*b*]flouranthene and benzo[*k*]flouranthene, and *m*- and *p*-xylene were analyzed together.

Statistical analysis was performed in GraphPad Prism 6. The significance of effect of each sediment and amendment (nitrate, crude oil and nitrate with crude oil) on the different enrichment

cultures was assessed by an unvaried analysis of variance (ANOVA) model with the Bonferroni post hoc test. A value of $p < 0.05$ was considered significant.

3.5. Results and discussion

3.5.1. Composition of bacterial communities

DGGE was used to allow preliminary insight into the structural diversity of bacterial communities present in the cultures, before in depth high-throughput sequencing characterization. The dendrogram generated from the DGGE profile of the 16s RNA gene fragments (Figure 3.6) shows a high variability between sub-samples, thus indicating that sequencing should be performed for each and not in composite samples obtained by combination of each enrichment culture with different sediment sub-sample.

Pyrosequencing of 16s RNA gene fragments was performed for all the 24 enrichment cultures with no amendment, only crude oil, only nitrate or both crude oil and nitrate (Figure 3.7). Sequencing results confirm the variability between sub-samples (differenced by the letters a, b and c). Both Aa and Ab sub-samples (active MV) present similar responses to the enrichment factors, while replicate Ac presents a very different community profile under all conditions, highlighting the natural variability of bacterial communities of deep-sea sediments, despite spatial proximity and similar geological context. This is confirmed in the analysis of bacterial OTUs which revealed differences between sub-samples a and b of active MV cultures in relation to Ac cultures, that actually grouped closer to all cultures of inactive MV sediments (Figure 3.8). Adonis analysis revealed a significant difference between these two clusters (adonis: $F_{1,23} = 5.398$, $R^2 = 0.197$, P =

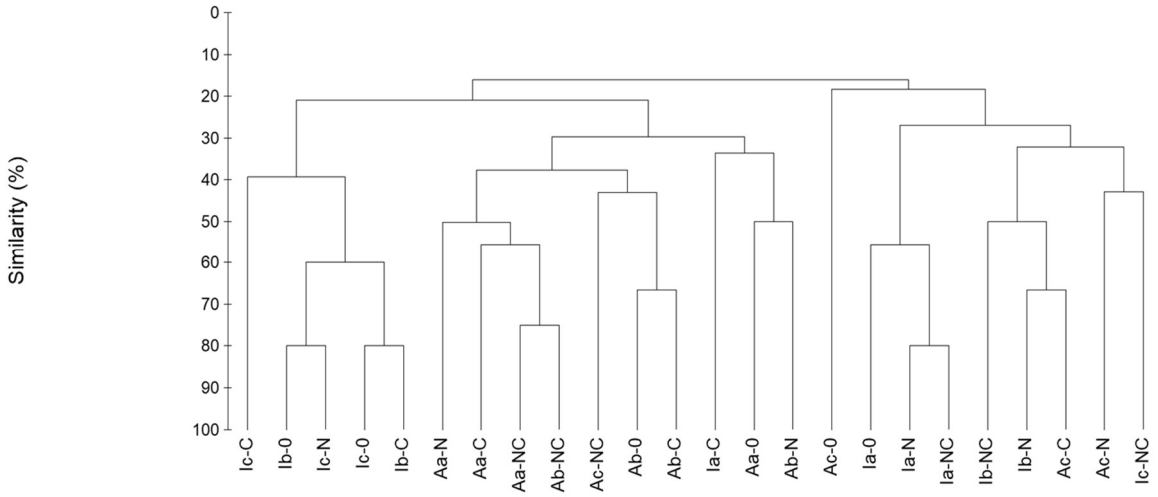

Figure 3.6 – Dendrogram representing the different enrichment cultures according to the structural similarity inferred by DGGE analysis of 16S rRNA gene sequences. Legend: A – active MV, I – inactive MV, 0 – no amendment, C – crude oil, N – nitrate and NC – nitrate and crude oil, and a, b and c indicate the different sub-samples for each experimental condition.

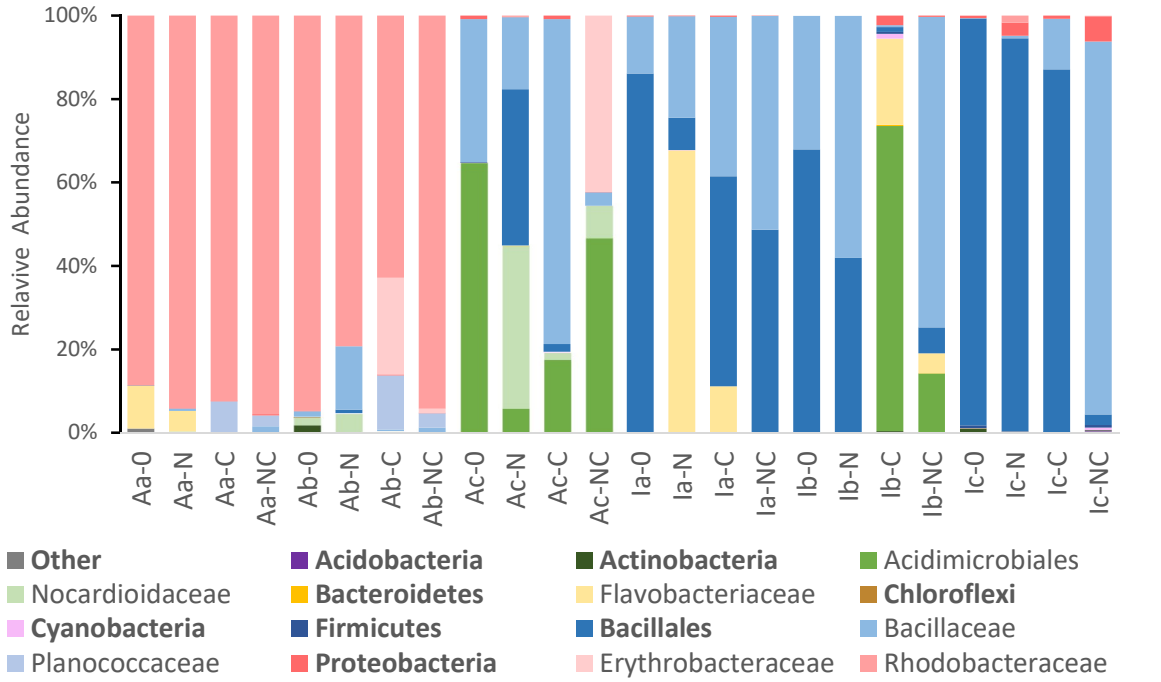

Figure 3.7 – Relative abundances of bacterial groups represented in anaerobic selective cultures. Bacterial communities were analyzed using 454 pyrosequencing of the V3V4 region of the bacterial 16S rRNA gene. Legend: A – active MV, I – inactive MV, 0 – no amendment, N – nitrate, C – crude oil, and NC – nitrate and crude oil. The letters a, b and c indicate the enrichment cultures with different sub-samples.

0.001). Amendments appears to also affect communities from the inactive MV. In the enrichment cultures without amendments all communities are almost entirely composed by Bacillales, with a variable representation of Bacillaceae. However, in enrichment cultures with amendments, other groups are better represented in the communities (except in Ia-N and Ib-C cultures). One-way ANOVA of the Shannon-Weaver diversity index for each group of sub-samples showed that neither the sediment origin nor the amendments had a significant effect on overall diversity of the selective cultures (SI Table 3.2).

3.5.1.1. Active vs. *inactive mud volcanoes*

Despite variability among sediment sub-samples, it is possible to detect differences between communities selected from active and inactive MV sediments. Under all tested conditions, communities from cultures of the a and b sub-samples of active MV are dominated by Alphaproteobacteria, representing over 79.0 % relative abundance, particularly members of the *Octadecabacter* and *Phaeobacter* genera. *Octadecabacter* are halophilic, psychrophilic and gas vacuolated aerobic or microaerophilic bacteria[386]. *Phaeobacter* are nitrite-reducing facultative anaerobes[387] and are known to form biofilms on biotic and abiotic marine surfaces[388,389]. *Phaeobacter* spp. were described as inhibited by hydrocarbons, such as aniline and naphthalene, but not to

cyclododecane[390], and were also related with diesel biodegradation[391]. In cultures Ab-C and Ac-NC, members of the Erythrobacteraceae family also exhibited relative abundances over 20.0 %. *Erythrobacter* was particularly well represented in the Ac-NC community. *Erythrobacter* includes marine aerobic chemoorganotrophs[392] and some species of aerobic anoxygenic phototrophs[393]. However, it is unlikely that those species were present in the enrichment cultures considering that they were incubated in the dark. Some *Erythrobacter* species are known to degrade crude oil[394,395] and more specifically PAHs[396,397]. However, at least one *Erythrobacter* species is known to positively affect hydrocarbon degradation by another bacteria without actually degrading the supplemented hydrocarbons, possibly either by metabolizing intermediaries from the hydrocarbon degradation by the petroleum-degrading bacteria, or by removing inhibitors from the medium[398]. *Erythrobacter* spp. can also require NaCl for growth[399,400], reduce nitrate[392,397] or operate denitrifying metabolism[397]. While most *Erythrobacter* spp. were isolated from surface seawater[393,399,400] some, particularly known PAH degraders, were isolated from the deep-sea[396,397]. Ac communities are largely composed of members of the Actinobacteria phylum, either belonging to the Acidimicrobiales order or the Nocardioidaceae family, and Bacillales order, with a large part belonging to the Bacillaceae family. Acidimicrobiales were identified in hydrocarbon contaminated sites and are often found in saline environments, such as marine environments[401], including deep-sea sediments[402]. The highest relative abundances within Bacillaceae in Ac communities were identified as *Bacillus* sp., *Thalassobacillus* sp. and other unspecified Bacillaceae members. *Bacillus* genus is known to include some of the best characterized hydrocarbonoclastic bacteria and anaerobic biosurfactant (BSF) producers, molecules known to improve PHs biodegradation[26,50,106,403]. In anaerobic metabolism, nitrate and organic compounds are often used as TEAs[404,405]. *Thalassobacillus* genus are moderately halophilic and are able of reducing nitrate to nitrite[406]. All communities from inactive MV sediments, except Ia-N and Ib-C, were dominated by Bacilalles. In most cultures, the

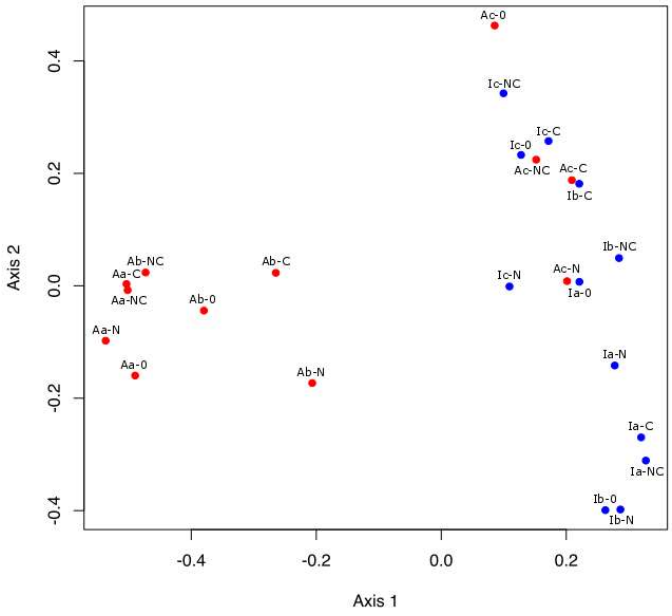

Figure 3.8 – Ordination showing the first two axes of the PCO analysis for OTU composition. Legend: A – active MV, I – inactive MV, 0 – no amendment, N – nitrate, C – crude oil and NC – nitrate and crude oil. The letters a, b and c indicate the different enrichment cultures with sub-samples.

majority of these OTUs belonged to the *Bacillus* genus. In four of the inactive MV cultures, Ia-N, Ia-C, Ib-C and Ib-NC, members of the Flavobacteriaceae family mostly belonging to the *Gramella* and, in the case of Ib-NC, to the *Maribacter* genera, were present with a relative abundance of at least 4.0 %. Cultures Ia-N, Ia-C and Ib-C presented high relative abundance (above 11.0 %) of *Gramella* spp. *Gramella* have been described as strict aerobes, unable of nitrate reduction or hydrogen sulfide production, with several of its members being reported in marine sediments[407,408]. Genome analysis of the species *G. forsetii* uncovered several genes involved in degradation of high molecular weight organic matter, indicating its ability to create blooms under feast conditions when the amount of high molecular weight dissolved organic matter increases[409]. *Maribacter* spp. corresponded to 4.3% of the OTUs associated with the culture Ib-NC. This genus is characterized by including strict aerobes and facultative anaerobes, that require Na^+ ions for growth[410,411]. They are also known to produce BSFs[412] and biofilms[413]. In the Aa-0 and Aa-N cultures the non-NRB, Flavobacteriaceae family member *Salinimicrobium* sp. corresponded to 10.3 % and 4.9 % of relative abundance, respectively. Members of this recently described genus[414] have previously been discovered in submerged sea sediments[415].

The composition of bacterial communities in sediments exhibit much higher short-scale spatial variation than pelagic communities[416]. As such, bacterial communities of deep-sea sediments are likely specific to certain micro-habitats. According to these results submerged sediments from active and inactive MVs present distinct microbial communities, even under identical enrichment factors. A major trend is the predominance of Alphaproteobacteria in active MV sediments and Bacilli in inactive MV sediments.

3.5.1.2. Response to enrichment factors

PCO did not show any clustering of cultures based on the enrichment factors (Figure 3.8). However, some response of the cultures to the enrichment factors were observed in low taxonomic categories. The presence of nitrate and/or crude appears to positively affect the Bacilli in the active MV cultures, except in Ac-NC. In the Aa and Ab communities the appearance of genera of the Planococcaceae family, particularly *Paenisporosarcina* and *Planomicrobium*, with a range of relative abundances of 7.5-12.9%, can be also related to the amendment with crude oil. However, in NC cultures these values drop to 2.6-3.4%. Thus, under fermentative conditions with crude oil, members of the Planococcaceae family are in metabolic advantage, but in the presence of nitrate, NRB may be positively selected. *Paenisporosarcina* are described as endospore-forming psychrotolerant strict aerobes[417] often found in cold environments, such as deep-sea sediments[418] or glaciers[419,420]. However, *Planomicrobium* spp. are known to include aerobic hydrocarbon-degraders[403,421,422]. Furthermore this genus includes species that able to form biofilms[423,424], tolerate

cold temperatures[425], reduce nitrate[424,425] and produce BSFs[422]. Members of the Erythrobacteraceae only reached abundances above 1.0% in selective cultures of active MV sediment amended with crude oil. In active MV cultures, amendment with crude oil (with or without nitrate) led to the decrease of the relative abundance of Actinobacteria, with the exception of the Ac-NC culture compared to Ac-N. It is unclear why there is this response, since Actinobacteria are a common taxonomic group found in marine sediments contaminated with PHs, both under aerobic[426,427] and anaerobic conditions[241]. Nitrate in cultures Ab-N, Ac-N and Ac-NC appears to have favored Nocardioidaceae which have been associated to PH degradation[428,429], in comparison to cultures Ab-0, Ac-0 and Ac-C. In inactive MV cultures, the presence of nitrate or crude oil led to an increase of Bacillaceae in detriment of others Bacillales, except for culture Ib-C. In cultures with both amendments, a cumulative effect was observed, reaching the highest relative abundances of Bacillaceae in all communities. Crude oil is also linked to an increase of the relative abundance of Acidimicrobiales and Flavobacteriaceae in cultures inoculated with inactive MV sediment. The groups being stimulated as a response to crude oil have all been previously associated to PHs biodegradation in sediments[145,281,430,431], except for *Paenisporosarcina*.

While bacterial communities are not always markedly affected by petroleum contamination[432], PHs often have an important effect on the structure of bacterial communities[165]. In the present work, a response of the microbial communities both to the presence of nitrate and PHs was observed. This indicates that bacterial communities of similar sediments with close spatial proximity could present different compositions due to environmental conditions. Since oil pollution in subsurface areas is often underestimated[433], and due to the difficulty of access and employment of bioremediation strategies in these environments, natural attenuation processes by bacteria are particularly important in the recovery of these environments[219]. In this sense, it is important to identify the main functional groups involved in PH degradation that can be used as biosensors for the contamination and indicators of recovery[212,434]. In the case of M. Ivanov active MV, Planococcaceae and Erythrobacteraceae can be suggested to act as biosensors for PHs, since members of both families were not detected in the absence of crude oil. For the inactive MV, Acidimicrobiales may be possible indicators of PH contamination.

The presence of different bacterial communities between conditions indicates that the OTUs associated with enrichment cultures containing crude oil are likely to play an active role, direct or indirect, in hydrocarbon biodegradation. That is, not all OTUs may directly degrade elements of crude oil but they can contribute to the adaptation of the communities to the presence of crude oil[237–239]. These bacteria can be responsible for the bottlenecks in the biodegradation process, by controlling nutrient availability, metabolic intermediaries, or fluxes and concentrations of degradation

products[197,240]. They can also be involved in several functions, like BSF production or capture of inhibitors to degradative bacteria[144,398].

3.5.1.3. General structure of bacterial communities

Biodegradation of PHs by indigenous bacteria present in contaminated environments is a ubiquitous process. However, the efficacy of the natural process and the preference for certain compounds varies with the location and structure of the bacterial community[217,218]. Indigenous hydrocarbonoclastic bacteria are usually present in small or residual quantities in marine environments. These communities quickly flourish upon petroleum or petroleum-based products contamination of the environment and PHs become available to be used as carbon and energy sources[209,217]. As such, PHs contamination imposes a strong selective pressure in bacterial communities[219]. This leads to a decrease of overall community diversity as a result of the toxicity of some PHs and the selectivity of specialized PHs degraders[219–223]. In general, bacterial diversity is inversely correlated with PHs concentration in contaminated environments[226]. In some PH contamination events, bacterial communities become so specialized that often one OTU can correspond to 90.0 % of the 16S rRNA gene sequences recovered[215,435]. In this study, all cultures presented only seven or less OTUs with relative abundance levels above 1.0%. In all cultures, except in Ac-N, Ac-NC, Ia-C and Ia-NC, a sole OTU dominated the community, mainly Bacillales in cultures obtained from sediments of inactive MV and Rhodobacteraceae in cultures from active MV sediments. Acidimicrobiales predominated in Ac-0 and Ib-C and Flavobacteriaceae in Ia-N. The most representative OTU in some cases corresponded to over 90.0% of relative abundance (Ab-0, Aa-N and Ic-0). In fact, an overall trend was that the highest relative abundance of a single OUT for all sub-samples was highest in the cultures without amendments (0) when compared to cultures amended with nitrate and/or crude (N, C and NC). Two exceptions are in Aa cultures, where Aa-N had the highest relative abundance of a single OUT, compared to other Aa cultures, and in Ib cultures which contradicts this trend and the dominant OUT presented higher relative abundance in Ib-NC and Ib-C cultures than in cultures with no amendment (0) or only nitrate (N). These results indicate that perhaps the pressure imposed by the anaerobic condition was responsible for single OTUs with high relative abundances, while nitrate and crude oil decreased the dominant OTUs relative abundances in favor of other OTUs that positively responded to the enrichment factors.

3.*5.1.4. Sporadic bacterial taxa*

Certain OTUs, with relative abundances over 1.0%, are sporadic, and do not configure a pattern associated with any of the enrichment factors. Nonetheless, most belong to taxonomic groups known to be involved in processes relevant to the context of this work. *Marinibacillus* spp. appeared

exclusively in culture Ab-N, with a relative abundance of 14.3%. Members of this genus are aerobic, endospore-forming, halophilic and psychrophilic or psychrotolerant and were isolated from deep-sea sediments[436,437]. *Stenotrophomonas* spp. were present in most of the cultures, albeit in low relative abundances, the highest being 1.3% in culture Ic-N. Certain members of this genetically diverse genus[438] are known to be highly adaptable and versatile[439] and to degrade aromatic and aliphatic PHs[440–442]. BSF production[442,443] and biofilm formation[441] is also documented. Despite most members being classified as strict aerobes, several are known to survive for long periods of time[444,445] or even grow under anaerobic conditions[446]. Due to these characteristics, they are often employed in bioremediation strategies[187,447,448]. *Anaerobacillus* spp. were identified in culture Ac-NC, with a relative abundance of 2.0% and in cultures Ia-0, Ib-0, Ia-NC, Ia-C and Ic-C with relative abundances below 0.2%. Members of this genus are strict anaerobes or aerotolerant, halotolerant or moderately halophilic, obligate or moderately alkaliphilic, diazotrophic and capable of fermentative or anaerobic respiration metabolism[449]. Furthermore, while not directly able to reduce Fe(III), they were described to form consortia with iron-reducing bacteria[450]. Members of the Endozoicimonaceae family of the Oceanospirillales order were identified in culture Ic-NC. Little is known about Endozoicimonaceae except that they can be aerobes or facultative anaerobes (fermentative), are halophilic and often associated to corals, jellyfish or other benthic marine invertebrates[451–454]. However, unspecified members of the Oceanospirillales order were associated with alkane and cycloalkane degradation and are known to bloom in marine environments, including at deep-sea, right after contamination by PHs[455,456]. Also in the context of oil contamination, these bacteria were found to be part of microbial biofilms[457,458]. In culture Ic-NC, *Variovorax* was identified with a relative abundance of 2.3%. This genus has associated with degradation of PAHs[459–461] and bioemulsifier production[462]. While most are aerobes[463], some are able of anaerobic growth using nitrate as a TEA[464,465].

3.5.1.5. Bacterial activity

Most of the identified OTUs belonged to genus or families associated to halophilic or halotolerant and psychrophilic or psychrotoletant bacteria. This was expected considering that the deep-sea is a cold saline environment. However, despite the anaerobic conditions imposed, many OTUs correspond to taxonomic groups not known to be able to perform anaerobic growth (e.g. *Gramella* and *Paenisporosarcina*). It is possible that anaerobic conditions in the vials were not strict enough, since in media selecting for NRB no chemical reductant was used. This was because the most used chemical reductants for NRB cultures (cysteine hydrochloride or sodium ascorbate) can be used as carbon sources[97,98] which was undesirable considering we were studding the effect of crude oil as sole carbon source. Although redox conditions corresponded to the clearance of resazurin, bacterial growth may have had its peak in the early days after inoculation when

microaerobic conditions were probably prevailing. Otherwise, bacteria had to tolerate a long period of oxygen deprivation. That may be the case for some spore forming bacteria such as *Marinibacillus*[437]. Due to the significance of supposedly non-spore forming obligate aerobes in some cultures, e.g. 67.6% *Gramella* spp. in Ia-N culture, it is also possible that either the strains are poorly characterized, or the genus characterization is not yet well defined. For example, members of the *Variovorax* genus were defined as being obligate aerobic[463] until a denitrifying species was recently discovered[465].

Many of the predominant OTUs belong to taxonomic groups of known PH degraders and/or BSF producers (e.g. *Phaeobacter* and *Bacillus*). While this is not an evidence of actual function, the fact that community structure responded to the addition of nitrate and/or crude oil indicates that the communities in these cultures take advantage of the amendments. In general, this is a good indicator of the biotechnological potential of the members of these communities.

3.5.1.6. Anaerobic enrichment

Overall, Firmicutes, almost exclusively composed of Bacilli, were the most represented group in inactive MV cultures, followed by phyla Bacteroidetes, Actinobacteria, Proteobacteria, while Chloroflexi, Acidobacteria, Cyanobacteria, and other Bacteria presented less than 1 % relative abundance in all cultures where they were present (SI Table 3.3). In cultures with sediments from active MV Proteobacteria was the predominant phylum, with most members belonging to the Alphaproteobacteria class, followed by Firmicutes, Actinobacteria and Bacteroidetes phyla, while Chloroflexi, Acidobacteria and other Bacteria were present in trace amounts in some cultures (SI Table 3.3).

The uncultured bacterial communities of the superficial sediments of the same sampling stations as used in this work were analyzed in a previous study, where DNA extraction was performed directly from the sediments[466]. Proteobacteria, the most abundant phylum in surface sediments of both MVs, with an average relative abundance of 69.4 % (± 2.8), presented higher relative abundances in the cultures Aa-0 and Ab-0 with sediments from 10 cm depth of active MV but greatly decreased in the cultures Ac-0 and all cultures containing sediments from the inactive MV (Ia-0, Ib-0 and Ic-0). Unlike superficial sediments where Gammapreobacteria was the most abundant class, followed by Alphaproteobacteria and Deltaproteobacteria, in the communities from enrichment cultures Aa-0 and Ab-0 Alphaproteobacteria was the predominant class. In inactive MV cultures Alphaproteobacteria presented averages of < 1% of relative abundance. The other classes also presented an average of < 1% of relative abundance for active and inactive MV enrichment cultures. Actinobacteria in surface sediments presented lower relative abundance, 7.9 % (± 2.0), than in Ac-0

cultures, which corresponded to 64.5 %, however in other cultures it either presented lower values, 3.7 % in Ab-0 and 0.96% in Ic-0, or is inexistent, in all other cultures without amendment. Of the following most abundant phyla in surface sediments, Chloroflexi and Gemmatimonadetes, only the former was detected in some enrichment cultures with sediment from active MV. Bacteroidetes and Firmicutes were very poorly represented in surface sediments, but were important groups in the bacterial communities from the enrichment cultures of MV sediments, especially the latter.

Overall, bacterial communities of surface sediment of both MVs have suffered important changes when compared to those of the enrichment cultures containing sediments from 10 cm depth. It is unclear if these differences are due to the difference in depth, due to the selective pressure applied by the anaerobic conditions or both.

Generally, Proteobacteria are known to be the predominant phylum in deep-sea sediments communities, with the following decreasing order of relative abundance, Gamma-, Delta- and Alphaproteobacteria[416]. So far, the most abundant classes of PAH-degrading bacteria found in deep-sea sediments are Gamma-, Alpha- and Betaproteobacteria[231]. Delta- and Gammaproteobacteria were the predominant classes found in anaerobic submerged sediments contaminated with crude oil from the Prestige oil spill[219]. However, in the present study, the three most predominant classes of Proteobacteria were Alpha-, Gamma- and Betaproteobacteria. Such a difference when compared to the global composition of deep-sea sediments is possibly the effect of the enrichment cultures. The Actinobacteria phylum can be often found in marine sediments[416]. It is known for its biotechnological potential, due to the production of biologically active metabolites[467]. As such, considerable efforts are being made to isolate and cultivate new marine Actinobacteria members with the purpose of finding novel metabolites of biotechnological interest, such as antibiotics[467]. Firmicutes are also not very common in deep-sea sediments, being more associated with coastal sediments[416]. Yet, this work shows that they appear to play an important role in inactive MV's bacterial community and to a smaller extent in active MV sediments.

3.5.2. Biodegradation of petroleum hydrocarbons

COD is a parameter used to indirectly measure the amount of organic compounds in liquids and is often used to assess the level of organic pollution and water quality[468]. As such it is not surprising that enrichment cultures with crude oil present higher COD values when compared to those that do not have it. No significant difference is observed between the different sediments, crudely indicating that either both communities could degrade PH and produce biomass and other metabolites at approximately the same rate, or that, more likely, differences are so small that could not be detected by the COD analysis.

GC-MS was used for a more detailed assessment of the biodegradation of PHs in each enrichment culture. The results represent the average values of all cultures (sub-samples a, b and c) of both sediment types (active or inactive MV). In some instances this led to large error bars (e.g. Figure 3.9) due to the variability between sub-samples in the degradation/production percentages (Figure 3.10), as already observed in the structure of bacterial communities.

3.5.2.1. n-*Alkanes*

n-Alkanes from low to medium molecular weight were more susceptible to biodegradation, with no statistically significant variation between cultures and abiotic controls being observed in alkanes above C_{13} (Figure 3.9). In cultures amended with only crude oil, heptane and octane were the preferred alkanes, independently of sediment origin. The percentage of degradation of C_7 and C_8 alkanes were of 40.25 % (± 7.16) and 19.99 % (± 3.06), respectively, for cultures with sediment from active MV, and 29.56 % (± 9.76) and 15.00 % (± 7.90) for cultures with sediment from inactive MV. It is possible that the presence of nitrate may have increased the versatility of the communities to metabolize PHs, leading to some sub-samples of the same cultures presenting production while others degradation of the same compound (e.g. Figure 3.10). With the production observed in some sub-samples (e.g. C_7 culture Ia-NC, Figure 3.10) likely being the result of degradation of longer alkanes[469]. Degradation percentages were significantly different from the control for *n*-alkane C_9 in A-C enrichment cultures (11.11 ± 3.01 %), and *n*-alkanes from C_{10} to C_{13} presented degradations percentages of 10.40 % (± 2.37), 10.28 % (± 2.27), 10.15 % (± 2.25) and 9.91 % (± 1.94),

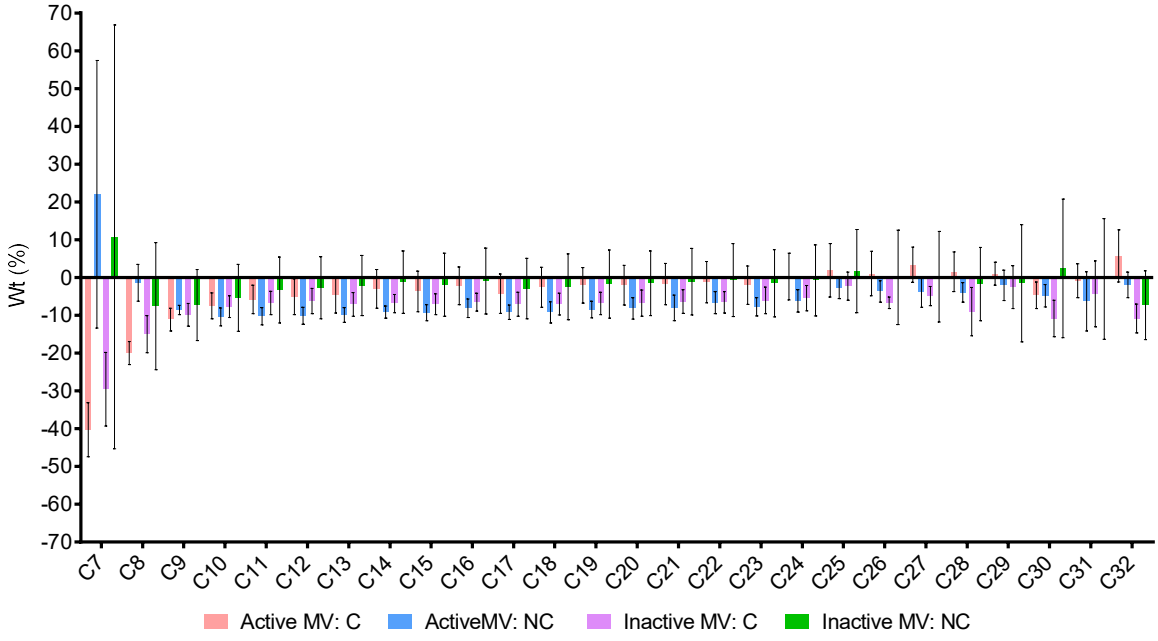

Figure 3.9 – Variation of each *n*-alkane (C_7 to C_{32}) relative weight between inoculated enrichment cultures and controls at the end of the last 5 weeks of incubation. MV – mud volcano, C – crude oil, N – nitrate, NC – nitrate and crude oil.

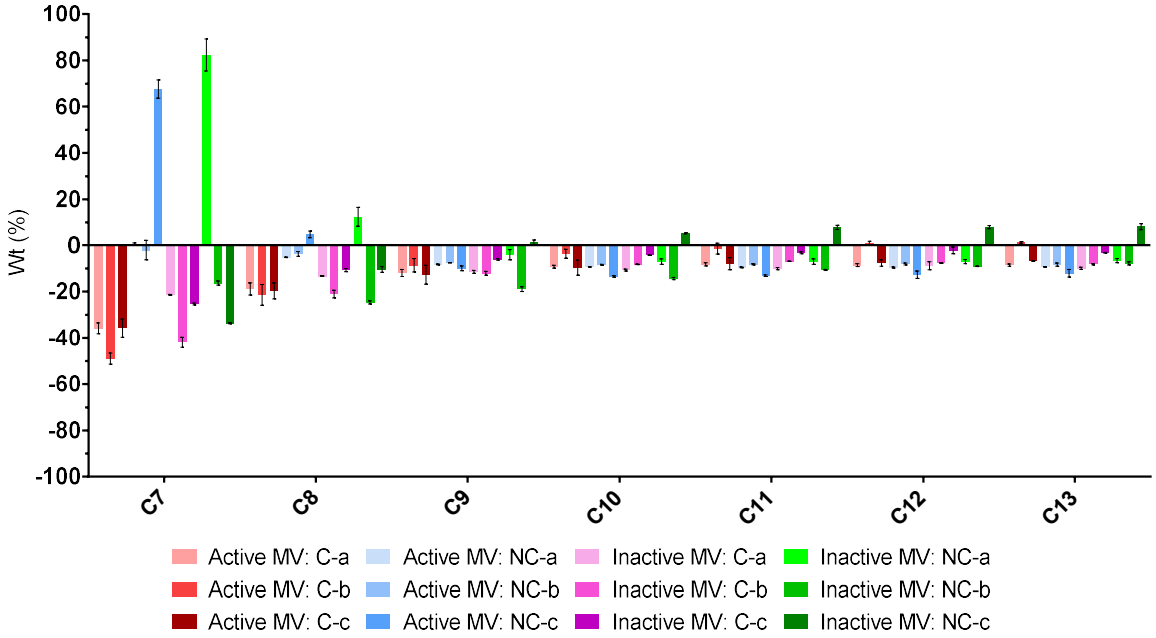

Figure 3.10 – Variation of each *n*-alkane (C_7 to C_{13}) relative weight between each enrichment culture inoculated with a different sediment sub-sample and controls at the end of the last 5 weeks of incubation. MV – mud volcano, C – crude oil, N – nitrate, NC – nitrate and crude oil. The letters a, b and c indicate the enrichment cultures with different sub-samples.

respectively, in A-NC enrichment cultures. Overall, in cultures with sediments from active MV degradation of a wider range of alkanes, from C_7 to C_{13}, was observed in comparison to cultures of sediments from inactive MVs in which only C_7 and C_8 were degraded. Degradation of *n*-alkanes larger than C_{13} was not observed. Since methane is one of the most represented hydrocarbons in MVs in Gulf of Cadiz[368] it is expectable that sediment bacterial communities are better equipped to degrade smaller *n*-alkanes. Communities from active MVs are more versatile degrading *n*-alkanes because although being the most abundant, methane is not the only hydrocarbon being released from the seeps[368]. Communities from inactive MV on the other hand, may be less versatile performing biodegradation of a wide range of PHs due to the scarcity of carbon sources naturally present in inactive MVs, most likely resulting in the development of very few specialists degrading the most abundant or the most recalcitrant hydrocarbons. The addition of nitrate to active MV cultures together with crude oil seems to shift the specialization of hydrocarbon degraders from *n*-alkanes < C_9 (including) to medium-size *n*-alkanes. This may be related with the decrease of members of the Planococcaceae family in active MV cultures.

3.5.2.2. Monoaromatic hydrocarbons

Of the analyzed MAHs (toluene, ethylbenzene, *m*-/*p*-xylene and *o*-xylene), toluene presented the highest degradation percentages, 31.32 % (± 7.03) in A-C culture and 25.65 % (± 5.98) in I-C cultures (Figure 3.11). As with the short-chained *n*-alkanes, the addition of nitrate led to more versatile biodegradation of MAHs between cultures with inactive MV sediments (SI Figure 3.1), particularly in I-NC cultures. This is unlike previous studies where nitrate amendment increased MAHs biodegradation[470]. Some of the toluene production observed in A-NC and I-NC cultures may be the result of PAHs degradation[319]. The other four MAHs were also significantly degraded in A-C cultures: 15.60 % (± 2.63) for ethylbenzene, 14.21 % (± 2.85) for *m*- and *p*-xylene and 16.38 % (± 1.40) for *o*-xylene. In I-C cultures *o*-xylene was also degraded 13.76 (± 3.96). As observed for the degradation of alkanes, the addition of nitrate did not significantly enhance degradation. Furthermore, the trend in biodegradation percentages is similar for A-C and I-C cultures with higher degradation of toluene, followed by *o*-xylene, ethylbenzene and *m*-/*p*-xylene.

3.5.2.3. Polycyclic aromatic hydrocarbons

From the 16 PAHs in the EPA watch list[385], indeno[1,2,3-*cd*]pyrene was not detected by GC-MS in the Light Arabian crude oil used in the selective cultures and benzo[*a*]pyrene, dibenz[*a,h*]anthracene and benzo[*g,h,i*]perylene were only detected in trace amounts. Statistically

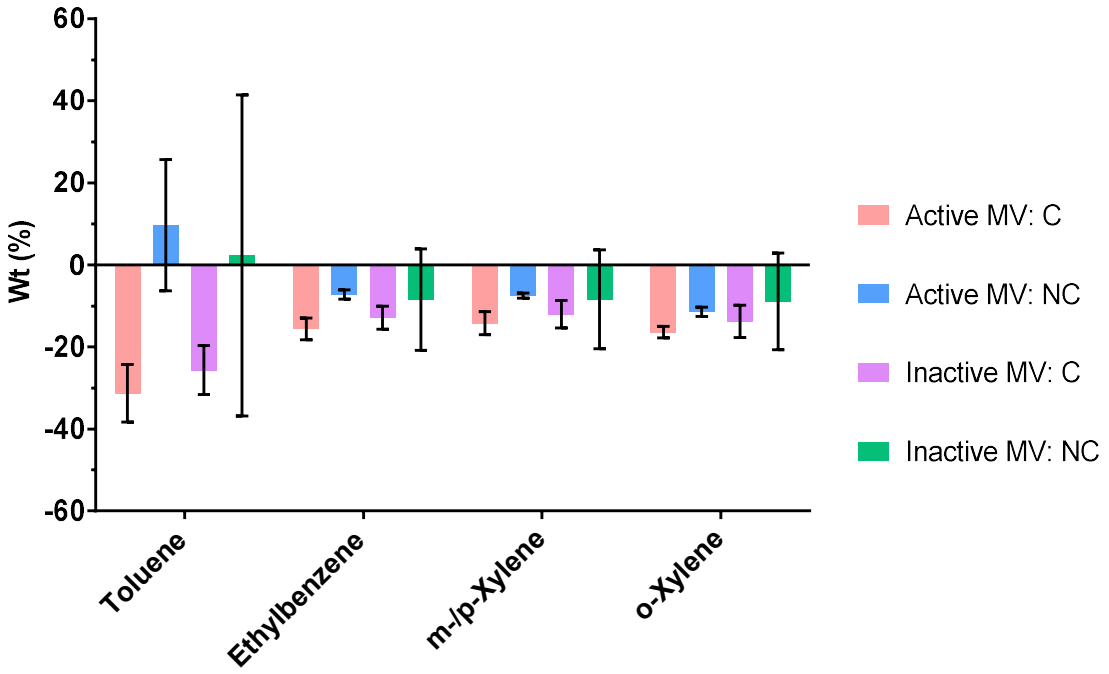

Figure 3.11 – Variation of toluene, ethylbenzene, *m*-/*p*-xylene and *o*-xylene relative weights between inoculated enrichment cultures and controls at the end of the last 5 weeks of incubation. C – crude oil, N – nitrate, NC – nitrate and crude oil.

significant biodegradation of flourene, phenanthrene, anthracene, pyrene and benzo[*b*]flouranthene plus benzo[*k*]flouranthene was observed in some cultures when compared to abiotic controls (Figure 3.12). The highest degradation percentages in all cultures were observed for anthracene with a maximum of 36.31 % (± 5.89) for I-NC cultures, followed by 33.62 % (± 14.09) for I-C, 28.75 % (± 5.78) for A-C and 24.56 % (± 4.59) for A-NC. Biodegradation of the other PAHs were observed in cultures with sediment from the inactive MV, with both C and NC, respectively: 11.92 % (± 6.78) and 12.85 % (± 2.85) for flourene; 15.29 % (± 8.39) and 16.16 % (± 3.50) for phenanthrene; and 18.11 % (± 9.27) and 12.50 % (± 7.09) for pyrene. With the exception of benzo[*b*]flouranthene plus benzo[*k*]flouranthene where degradation was only significant for I-C cultures, 14.66 % (± 7.11). Except flourene, which has two benzene rings, all of these PAHs have three or four benzene rings.

Low molecular weight PAHs are dissolved in water while larger molecules tend to adsorb to suspended solid matter and sediments[146,165]. Aromatic compounds with medium and high molecular weight are known to be the most persistent compounds in sediments[165]. It is possible that these are the most predominant PAHs in submerged sediments of Gulf of Cadiz, probably originating from the heavy maritime traffic in the area. No significant differences between presence and absence of

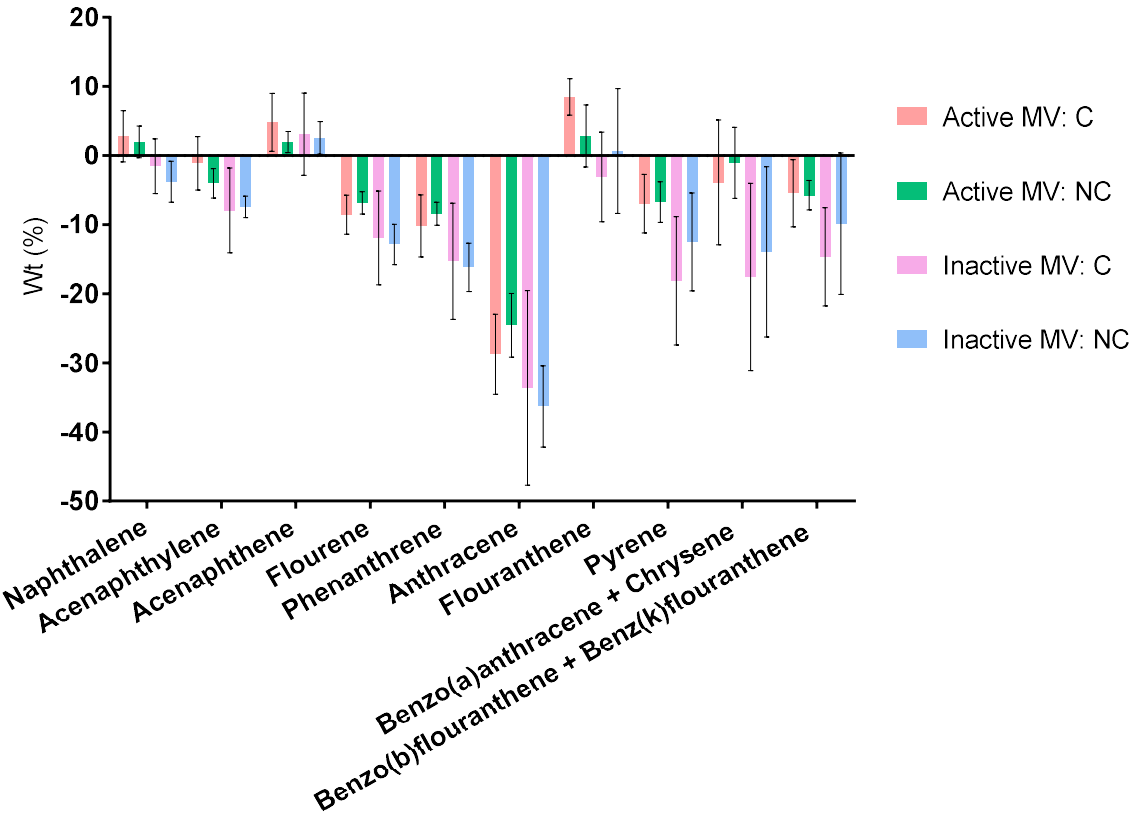

Figure 3.12 – Variation of each PAH relative weight between inoculated enrichment cultures and controls at the end of the last 5 weeks of incubation. MV – mud volcano, C – crude oil, N – nitrate, NC – nitrate and crude oil.

nitrate were detected regarding PAHs degradation. However, the capacity for PAH biodegradation in the bacterial communities in the selective cultures was significantly determined by the sediment origin (active or non-active MV). Communities from inactive MV sediments were able to degrade a much wider range of PAHs than those from active MVs.

3.5.2.4. General considerations

With the purpose of gaining insights on bacterial metabolism in the enrichment cultures HPLC was used to quantify the production of organic acids (acetic, butyric, propionic and valeric acids) resulting from the degradation of PHs under anaerobic conditions[277,471]. However organic acids were not detected for any culture, likely due to the low metabolic rates characteristic of anaerobic environments.

Overall, bacterial communities from cultures with sediments from active MV presented higher degradation percentages for *n*-alkanes and MAHs, while those from cultures with sediments from inactive MV were better at degrading PAHs. Most likely this is related with the predominance of Alphaproteobacteria in active MV cultures of and Bacillales in inactive MV cultures. Not much is known regarding the predominant Alphaproteobacteria genera in the enrichment cultures. Some *Phaeobacter* members are known degraders of alkanes under aerobic conditions[210] and some members of *Octadecabacter* although not usually associated to PHs biodegradation, are known to carry the alkB gene, involved in alkane degradation[314]. Bacillales and more specifically *Bacillus*, are known to be versatile PH degraders[472] which contradicts the low metabolic versatility observed for PH degradation by bacterial communities from inactive MV cultures.

Deep-sea bacterial communities, particularly in areas where natural seepage occurs, are known to be adapted to PHs[165,215]. While it is expectable for communities from active MV sediments to be able to efficiently degrade small PHs like those seeping at the MVs, it is not clear why communities from inactive MVs are better at degrading PAH. It is possible that these communities have adapted to degrade the most recalcitrant PHs, known to accumulate in sediments[146,165], since less complex ones are more readily exhausted. Nitrate addition as TEA had a negative impact on the overall degradation of small *n*-alkanes and MAHs, improved degradation of medium-chained *n*-alkanes and had no effect on the degradation of PAHs. An important bioremediation strategy to improve *in situ* bioremediation of oxygen deprived environments is the addition of alternative TEAs in order to stimulate more energetically favorable metabolisms involved in PHs degradation[169,255]. This is of particular interest in aquatic or submerged environments since most TEAs, such as sulfate and nitrate, used in anaerobic metabolism are more soluble than O_2 [248,253]. TEAs receive electrons that are transferred from electron donor substrates (e.g. hydrocarbons) by bacteria, while in the

process generating energy necessary for the cell activity and growth[256]. Nitrate could be used in bioremediation strategies in areas contaminated with *n*-alkanes-rich products, such as light crude oil, but most likely it would have limited results and further strategies would needed for the clearance of other PHs.

3.6. Conclusion

This work allowed an in-depth analysis of bacterial community of sediments from MV with different types of activity and their responses to the presence or absence of nitrate and/or crude oil. It was possible to identify the main players in PHs degradation in the deep-sea MVs from gulf of Cadiz. The bacterial communities of active and inactive MVs sediments from the Gulf of Cadiz reacted to the presence of crude oil and/or nitrate differently depending on the origin of the sediment. Cultures with inocula from the active MV showed higher relative abundance of Alphaproteobacteria, while those with inocula from the inactive MV are richer in Bacilli. Planococcaceae and Erythrobacteraceae are characteristic of crude oil contamination in active MV cultures and Acidimicrobiales in inactive MV cultures. In general, communities from active MV cultures were able to degrade a wider range of *n*-alkanes and MAHs than those from inactive MV cultures. However the later were able to degrade more PAHs than the communities from active MV cultures. Nitrate addition did not have a marked effect on the degradation ability of bacterial communities and induced the degradation on medium size *n*-alkanes. To understand which hydrocarbonoclastic bacteria can be of importance in PH bioremediation, culture-based studies are still necessary to assess the catabolic abilities of these microorganisms, and ecological studies are necessary to understand the *in situ* performance of particular microbial groups.

3.7. Acknowledgments

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3.8. Supplementary information

SI Table 3.1 – List of the 15 most abundant OTUs (in italic) and other OTUs associated with taxonomic groups relevant to the scope of this work. The table includes the OTU-numbers (OTU); taxonomic assignment generated by QIIME; GenBank accession number (Accession No.), sequence similarity of the closest matches with our representative OTU sequences (Seq. Sim.) and the source of these organisms (Source/Context).

OTU	Phylum	Class	Order	Family	Genus	Accession No.	Seq. Sim.	Source/Context
1	*Firmicutes*	*Bacilli*	*Bacillales*	*Unclassified*	*Unclassified*	*DQ448751*	*100*	*Marine sediment, Republic of Palau*
2	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Octadecabacter*	*KT185203*	*100*	*Continental shelf sediment from Cabo Frio upwelling region, Brazil*
3	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Phaeobacter*	*KJ862101*	*100*	*Sediments of marine hydrothermal vent fields, Lucky Strike vent field, Atlantic Ocean*
4	*Actinobacteria*	*Acidimicrobiia*	*Acidimicrobiales*	*Unclassified*	*Unclassified*	*KM095057*	*100*	*Petroleum-contaminated soil, Argentina*
5	*Firmicutes*	*Bacilli*	*Bacillales*	*Unclassified*	*Unclassified*	*KJ756132*	*100*	*Deep-sea Gulf of Mexico sediment enriched with ANS 521 artificially weathered crude oil as sole C-source*
6	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*DQ079008*	*100*	*Deep-sea hydrothermal mat-covered sediment, Guaymas Basin, Pacific Ocean*
7	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Gramella*	*JX854241*	*99*	*Coastal sediment, Sylt (West Beach), North Sea*
8	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Thalassobacillus*	*JN791393*	*99*	*Marine sediment, Ximen island, China*

OTU	Phylum	Class	Order	Family	Genus	Accession No.	Seq. Sim.	Source/Context
9	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*KJ575022*	*100*	*Deep-sea sediment, Andaman Sea. Indic Ocean*
10	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*MF045087*	*100*	*Ocean sediment, Bohai Bay, China*
11	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Nocardioidaceae*	*Unclassified*	*HM222657*	*100*	*Deep-sea sediment*
12	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Erythrobacteraceae*	*Erythrobacter*	*KP265725*	*100*	*Cultures of marine sediment (Atlantic Ocean) amended with*
13	Firmicutes	Bacilli	Bacillales	Planococcaceae	Paenisporosarcina	FJ649498	100	Amsterdam Mud Volcano sapropel sediment, East
14	Firmicutes	Bacilli	Bacillales	Bacillaceae	Marinibacillus	LC095014	100	Deep-sea sediment, Sagami Bay, Japan
15	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Salinimicrobium	AB274735	99	Intestine of Senegal sole (fish) mass reared in Cadiz, Spain
17	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Variovorax	KY945896	100	Soil
18	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Maribacter	MF039067	99	Seamount near Yap Trench, Pacific Ocean
19	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	KT933191	100	Enrichment cultures of submerged Arenella harbor sediment (Italy) with diesel as
22	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planomicrobium	DQ356977	100	Deep-sea sediment, Okinawa Trough, Pacific Ocean
27	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Endozoicimonaceae	Unclassified	GU118396	100	Gorgonia ventalina (coral), Panama

OTU	Phylum	Class	Order	Family	Genus	Accession No.	Seq. Sim.	Source/Context
84	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Unclassified*	*AB167049*	*100*	*Deep-sea sediment, Kumano nada mud volcano No. 5, Nankai*
90	*Firmicutes*	*Bacilli*	*Bacillales*	*Unclassified*	*Unclassified*	*FM992834*	*100*	*Deep-sea sediment, Eastern Mediterranean*
109	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Phaeobacter	AB948561	100	Subsurface methane hydrate-bearing sediment, Ulleung
110	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*KT074379*	*100*	*Continental shelf sediment, Arabian Sea*
126	Firmicutes	Bacilli	Bacillales	Bacillaceae	Anaerobacillus	KY775658	99	Saline alkaline soil, Lake Natron, Tanzania

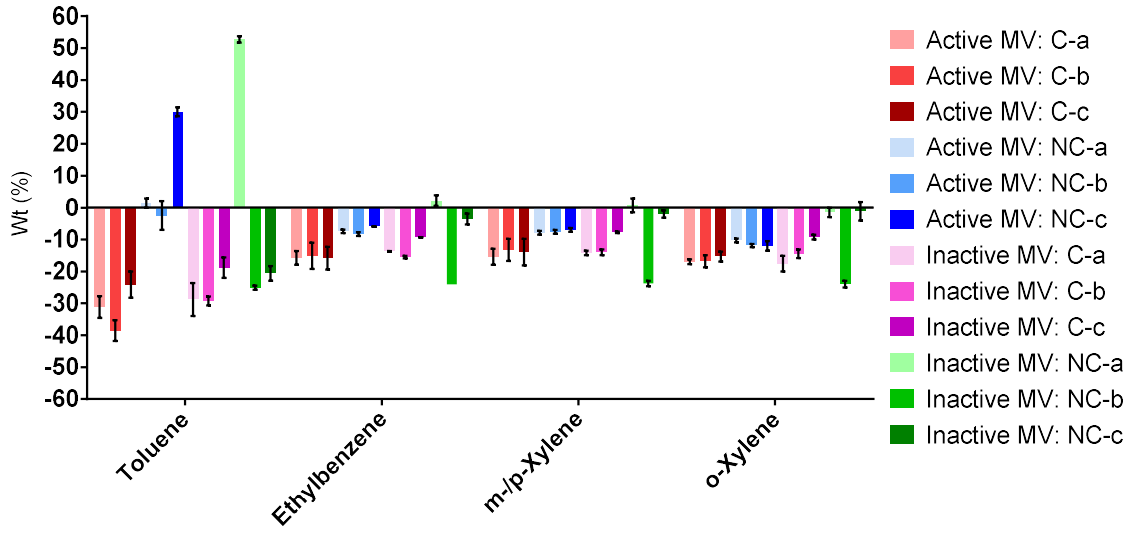
SI Table 3.2 – Shannon-Weaver diversity index for each enrichment culture sequenced. Legend: A – active MV, I – inactive MV, 0 – no amendment, N – nitrate, C – crude oil and NC – nitrate and crude oil. The letters a, b and c indicate the different enrichment cultures with sub-samples.

	Aa-0	**Ab-0**	**Ac-0**	**Ia-0**	**Ib-0**	**Ic-0**
Shannon diversity index (H')	0.524	0.275	1.042	0.414	0.646	0.146
	Aa-N	**Ab-N**	**Ac-N**	**Ia-N**	**Ib-N**	**Ic-N**
Shannon diversity index (H')	0.398	0.699	1.471	0.868	0.771	0.393
	Aa-C	**Ab-C**	**Ac-C**	**Ia-C**	**Ib-C**	**Ic-C**
Shannon diversity index (H')	0.615	0.962	0.920	1.241	0.813	0.423
	Aa-NC	**Ab-NC**	**Ac-NC**	**Ia-NC**	**Ib-NC**	**Ic-NC**
Shannon diversity index (H')	0.755	0.291	1.048	0.990	0.871	0.573

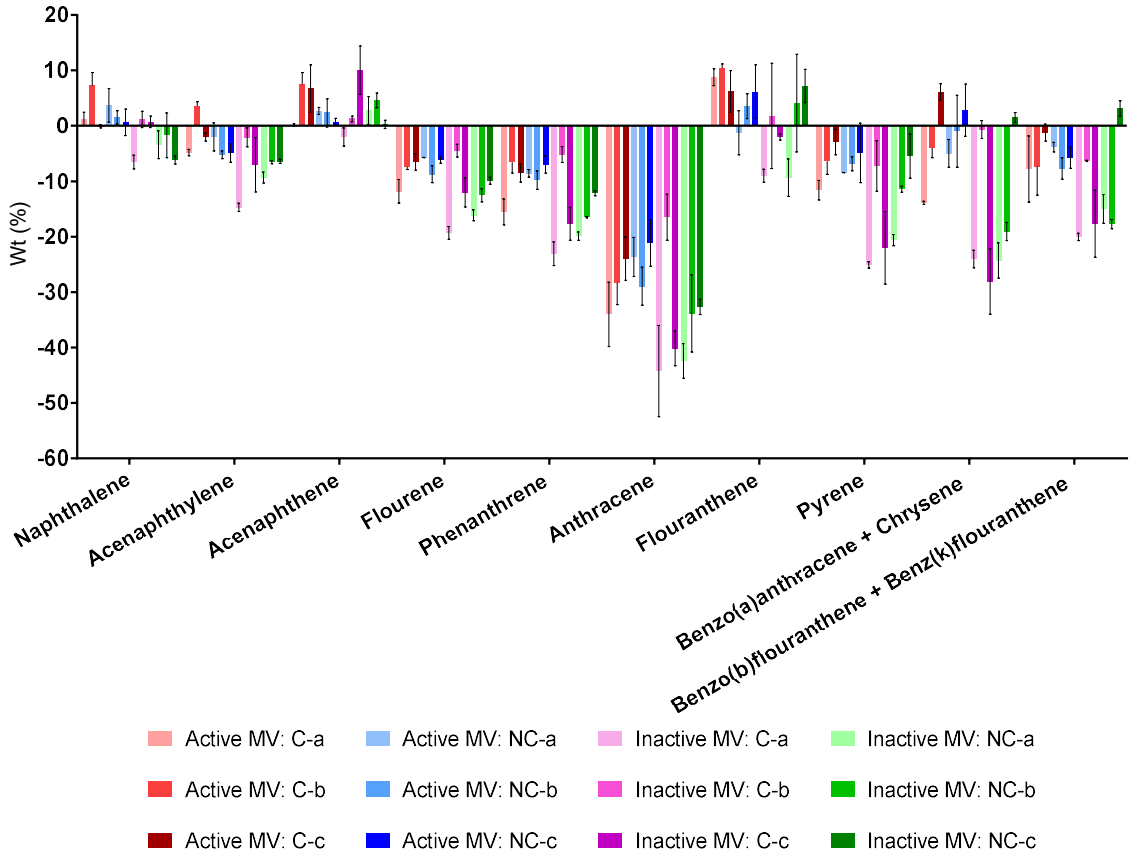
SI Table 3.3 – Relative abundance (%) of bacterial phyla in each enrichment culture. Legend: A – active MV, I – inactive MV, 0 – no amendment, N – nitrate, C – crude oil and NC – nitrate and crude oil. The letters a, b and c indicate the different enrichment cultures with sub-samples.

Phyla	Aa-0	Aa-N	Aa-C	Aa-NC	Ab-0	Ab-N	Ab-C	Ab-NC	Ac-0	Ac-N	Ac-C	Ac-NC
Other bacteria	0.84	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.04	0.00	0.03
Acidobacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00
Actinobacteria	0.00	0.25	0.02	0.00	3.77	4.73	0.09	0.00	64.54	44.82	19.30	54.40
Bacteroidetes	10.31	4.86	0.00	0.00	0.03	0.00	0.31	0.08	0.00	0.04	0.00	0.00
Chloroflexi	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.04	0.03	0.00	0.00	0.00
Cyanobacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	0.07	0.61	7.52	4.17	1.35	16.05	13.35	4.58	34.45	54.73	79.70	3.22
Proteobacteria	88.60	94.17	92.46	95.83	94.82	79.22	86.25	95.31	0.80	0.39	0.82	42.35

	Ia-0	Ia-N	Ia-C	Ia-NC	Ib-0	Ib-N	Ib-C	Ib-NC	Ic-0	Ic-N	Ic-C	Ic-NC
Other bacteria	0.00	0.04	0.00	0.03	0.00	0.00	0.14	0.00	0.06	0.15	0.00	0.25
Acidobacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.22
Actinobacteria	0.00	0.08	0.00	0.00	0.00	0.00	73.41	14.23	0.96	0.00	0.02	0.22
Bacteroidetes	0.00	67.63	11.15	0.06	0.04	0.06	20.92	4.83	0.06	0.06	0.10	0.00
Chloroflexi	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cyanobacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00
Firmicutes	99.70	32.11	88.54	99.84	99.96	99.94	2.06	80.69	98.31	94.93	99.11	92.53
Proteobacteria	0.25	0.11	0.31	0.06	0.00	0.00	2.30	0.25	0.58	4.83	0.72	6.21



SI Figure 3.1 – Variation of toluene, ethylbenzene, *m*-/*p*-xylene and o-xylene relative weights between each enrichment culture inoculated with a different sediment sub-sample and controls at the end of the last 5 weeks of incubation. C – crude oil, N – nitrate, NC – nitrate and crude oil. The letters a, b and c indicate the different enrichment cultures with sub-samples.



SI Figure 3.2 – Variation of each PAH relative weight between each enrichment culture inoculated with a different sediment sub-sample and controls at the end of the last 5 weeks of incubation. C – crude oil, N – nitrate, NC – nitrate and crude oil. The letters a, b and c indicate the different enrichment cultures with sub-samples.

Chapter 4

CHAPTER 4

Isolation of facultative anaerobic hydrocarbonoclastic biosurfactant-producing bacteria from subaquatic sediments

Patrícia M. Domingues, Vanessa Oliveira, Adelaide Almeida, Luísa S. Serafim,

Newton C. M. Gomes, Ângela Cunha

4.1. Abstract

Bacterial communities from subseafloor sediments are still poorly characterized with only few bacteria being culturable, despite these environments being known to have diverse communities with high biotechnological potential. This work aims at characterizing the populations of culturable hydrocarbon degrading and biosurfactant (BSF) producing bacteria from deep-sea (mud volcanos from Gulf of Cadiz) and estuary (Ria de Aveiro) submerged sediments. The bacterial communities from submerged sediments grown in anaerobic selective cultures with crude oil as carbon source presented communities rich in nitrate-reducing bacteria. From selective cultures with deep-sea and estuary sediments seven and six hydrocarbon-degrading BSF-producing isolates were isolated, respectively. These were identified by sequencing of 16S rRNA gene fragments as belonging to *Psedomonas*, *Bacillus*, *Ochrobactrum*, *Brevundimonas, Psychrobacter, Staphylococcus, Marinobacter* and *Curtobacterium* genera. BSF production by the isolates was tested by the hemolytic activity, drop collapse test, atomized oil assay, surface tension and emulsification index measurements. Although none of the isolates tested were positive for hemolytic activity and drop collapse test, they were all able to produce BSFs under aerobic and anaerobic conditions, except for isolates DS27 and DS230 which only produced BSF under aerobic conditions. Overall all the isolates justify further studies and present potential to be applied as biorremediation agents for decontamination of petroleum in marine sediments or for microbial enhanced oil recovery strategies.

4.2. Key words

Deep-sea; estuary; atomized oil assay; petroleum hydrocarbons; anoxic.

4.3. Introduction

Biosurfactants (BSFs) are amphiphilic molecules that alter the surface and interfacial tensions promoting the dispersion of one phase into the other[473]. They can also be considered bioemulsifiers when they promote the formation of stable emulsions[4,5]. BSFs can be produced by several microorganisms, including bacteria, with diverse ecological purposes[474], including the

increase of bioavailability of surface-bound and hydrophobic substrates, such as petroleum hydrocarbons (PHs), via direct interfacial contact and pseudosolubilization[9,11]. This characteristic is particular advantageous for application of BSFs in PHs bioremediation strategies or microbial enhanced oil recovery (MEOR)[9,19]. Since in many cases the contaminated environments in which bioremediation is to be applied or the oil wells requiring MEOR strategies present anaerobic conditions, anaerobic BSF producing bacteria would be the most adequate[475]. Anaerobic BSF production may also be an advantage at industrial level due to decreased foam formation[75]. However to the moment only a few bacteria are known to produce BSFs under anaerobic conditions, most of which belonging to the *Bacillus* and *Pseudomonas* genera[475].

Hydrocarbonoclastic bacteria are characterized by being able to metabolize one or more PHs by using them as carbon and energy sources, usually preferring these substrates over others[264–266]. This group comprises tenths of genera that belong to Proteobacteria, especially Alpha-, Beta- and Gammaproteobacteria classes, and also Actinobacteria and Firmicutes phyla, with new members being continuously identified[197,215,223,267,268]. These bacteria are ubiquitous and can be found both in aerobic and anaerobic environments[143,242,283]. They are usually present in very low abundance in pristine or low-contaminated aquatic environments and bloom after contamination with PHs[143,208,212]. Natural anaerobic biodegradation of PHs occurs primarily in deep subsurface oil reservoirs[278], in the deep ocean near natural seeps[279,280], and in most microaerobic or anaerobic environments contaminated with PHs[218,263,281].

Sediments represent some of the most phylogenetically diverse microbial habitats[476]. Submerged sediments are rich sources of bacteria with diverse biotechnological abilities. Several studies have already explored some of these environments in search of hydrocarbonoclastic or BSF-producing bacteria, or the community capacity for hydrocarbon degradation[209,242,243]. Marine submerged sediments are often anoxic below a thin upper layer whose width is generally directly correlated with water depth[477]. In the upper layers of submerged sediments there is still enough O_2 for aerobic metabolism by bacteria due to oxygen diffusion from the water column, the activity of macrobenthos responsible for bioturbation and, in sediments exposed to light, oxygenic photosynthesis[478]. However only the upper millimeters to centimeters are permanently oxygenated. A vertical stratification of predominant metabolism is often observed in submerged sediments and depends mainly on the availability of carbon source and terminal electron acceptors (TEAs), which are often introduced from the sediment surface and transported downwards. Since microorganisms tend to sequentially consume more metabolically favorable TEAs first, redox potentials decrease with depth resulting in a pattern known as redox zonation or stratification[256,357,479]. In the oxidized layer of costal sediments, aerobic and nitrate-reducing bacteria (NRB) predominate, and their relative abundance decreases with depth. The prevailing metabolism at the intermediate transition layer is

sulfate reduction. However, more than one type of metabolism can be observed at any layer, with different TEAs being used depending on their availability[219].

The presence of PHs in submerged sediments in concentrations that exceed natural remineralization capacity stimulate bacterial aerobic metabolism leading to a faster depletion of dissolved oxygen in upper sediment layers and consequent thickening of the anaerobic zones beneath, when compared to non-contaminated sediments[209,246,277]. Oxygen depletion will affect the abundance and diversity of macro- and micro-benthic populations[293]. The depth at which anaerobic conditions are observed in the subsurface sediment layers, often depends on the organic matter sedimentation rate, particularly in deep-sea, and can vary between a few millimeters below seafloor to a depth of several centimeters[361].

The lack of light, slow currents, scarce sediment accumulation, low supply of organic matter, low temperature (< 5°C), high pressure (> 10 MPa) and high salinity (34.3-35.1 g/L) make the deep-sea an extreme environment. These physical conditions are relatively stable in the extension of deep-sea floor[356,357] and since the single source of organic carbon for the vast majority of the deep-sea is the deposition of detritus from surface waters, most of the deep-sea is characterized by low energy, productivity and biological rates[355]. However metabolic activity and biomass is higher in locations where seepage of PHs and other compounds occurs from within deep sediment layers to the seabed, such as hydrothermal vents and cold seeps[250,358]. Cold seeps are geologically diverse ecosystems, including mud volcanoes (MVs), associated with emissions of methane, other PHs and often reduced sulfate from the subsurface found at active and passive margins in the deep-sea[362]. As such, the deep-sea can potentially be a hidden reservoir of bacterial diversity and novel metabolic activity, with many new bacteria still being discovered[369]. Generally, bacterial communities from the deep-sea are functionally versatile, highly diverse and adaptable to alterations in organic matter inputs[356].

Surface deep-sea sediments are known sinks for aliphatic and aromatic hydrocarbons of both natural and anthropogenic origin[231,365]. Furthermore, deep-sea surface sediments in which PHs are present are enriched in functional genes associated with aerobic and anaerobic degradation of hydrocarbons, in comparison with adjacent sediments without PHs[165,262]. This indicates that deep-sea bacterial communities, particularly in areas where natural seepage occurs, are adapted to PH availability[215].

Estuarine systems are characterized by dynamic exchanges of nutrients and pollutants with the ocean and river waters and are known sinks for particulate organic matter and contaminants[293], with PHs being one of the most common contaminants[162]. Intertidal wetlands are usually affected by low tidal and wave energy which allows for PHs to enter these ecosystems but makes it difficult for them to be washed out, particularly after being trapped in vegetation and sediments which are usually

fine-grained[480]. Bioturbation, the displacement of sediments by fauna and flora, can contribute to further bury PHs to microaerobic or anaerobic environments[480]. PHs contamination of estuaries quickly leads to a reduction of redox potential in sediments, even those that are not constantly submerged[479]. Thus PH presence in estuary sediments is often associated with microaerobic or anaerobic conditions[481]. Estuary sediments are rich in diverse and adaptable bacterial communities, known to include hydrocarbon-degrading bacteria, especially in contaminated areas[205,210,482].

The aim of this work was to isolate and identify bacteria with high biotechnological potential, from subaquatic sediments, in particular from the deep-sea and estuarine sites, which could be later used in anaerobic bioremediation and MEOR strategies. A second objective was to identify key players in anaerobic natural hydrocarbon biodegradation in estuarine and marine ecosystems. As such, anaerobic selective cultures were prepared with only crude oil as carbon source and submerged sediments as inoculum. Bacteria isolated from these cultures were subsequently identified and characterized in terms of BSF production under aerobic and anaerobic conditions.

4.4. Materials and Methods

4.4.1. Study area and sampling

4.4.1.1. Estuarine system, Ria de Aveiro

The shallow estuary-coastal lagoon system of Ria de Aveiro is located in north Portugal at approximately 40.7° N, 8.7° W (Figure 4.13). With a complex topography, the estuary is characterized by a single tidal channel connected to the Atlantic ocean that branches out into several smaller channels[483].

All samples were collected on the 25th February 2013 at an unvegetated intertidal sampling site (40°37'32.18" N, 8°44'09.12" W) located at the east margin of Mira channel, one of the main branches of the estuarine system of Ria de Aveiro. The sampling site is located in the proximity of the commercial port of Aveiro and several small harbors used by leisure and artisanal fishing boats. This area has been previously characterized as contaminated with PHs, both aliphatic and aromatic and at this particular site, surface sediments present contents of 5.81 % organic matter, 36.61 % moisture, 81.99 % fines and mud sediment texture[145]. Another study characterizing prokaryote distribution, activity and utilization of carbon sources in these sediments reported a decrease of bacterial abundance and heterotrophic activity with depth[484].

Sediment samples were collected with a cylindrical steel corer, with 8 cm diameter and 55.5 cm length. Three replicates were collected within an area of 1 m^2. Each sediment column

approximately presented an upper 5 cm layer composed of fine sand sediment, followed by a layer of dark muddy sediments from 10 up to 15 cm depth (M) and a medium-grained sandy sediment layer at lowers depths (S1). In two of the cores, a medium-grained sand sediment layer with an orange coloration was also observed in between 15 to 30 cm depth (S2). The sediment samples were transported to the laboratory in separated plastic bags that were sealed and kept at -4 °C until separation of the layers M, S1 and S2 and further analysis. Sediments S1 and S2 were pooled together and treated as sandy sediment (S). Corresponding S and M strata of each replicate core were pooled together to obtain composite samples.

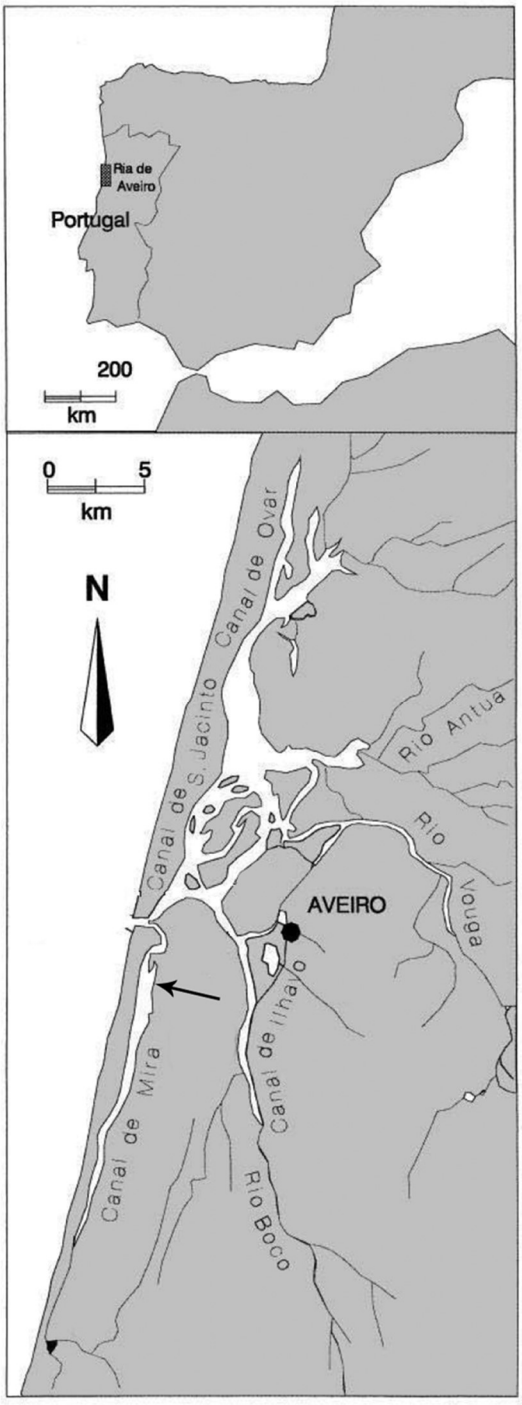


Figure 4.13 – Ria de Aveiro (Portugal). Arrow indicates sampling site.

4.4.1.2. Deep-sea, Gulf of Cadiz

MVs are structures commonly found in the accretionary wedge that crosses the Gulf of Cadiz (AWGC; Figure 3.5), at depths between 200 m and 5000 m[370,371]. Here, natural seepage of gas and hydrocarbons, particularly methane, sustains an ecosystem based on chemosynthetic energy[368]. Furthermore, in the Gulf of Cadiz, MVs occur in areas of intense maritime traffic, hence subjected to anthropogenic inputs of oil hydrocarbons and highly exposed to the risks related to shipping accidents[372].

The samples used in this study were collected during the SWIMGLO/Transflux M86/5 cruise onboard the RV Meteor[373] at the Mikahail Ivanov MV located on the AWGC and formed by several craters. Samples were collected at the northwest crater, station 348, which presents no indication of seepage activity, and at the active southeast crater, station 329 (Table 3.5). Sampling was conducted with a box corer[373]. Sub-samples of 0.5-1 g of subsurface sediments (10 cm below seafloor) were collected, immediately deep-frozen (-80 °C), kept on dry ice during shipping, and stored at -80 °C until further analysis.

4.4.2. Anaerobic selective cultures

The general procedure for preparation of anaerobic selective cultures involved the use of a minimum mineral medium, with light Arabian crude oil as sole carbon source, and inoculation with sediments from either estuarine or deep-sea sediments. Resazurin was used as a redox indicator and cycloheximide as an anti-fungal agent. Preparation of the anoxic media was performed as described in Plugge (2005) and Wolfe (2011) [97,374]. Aliquots of crude oil were UV-sterilized for 4 hours, added to the culture vials and autoclaved. Except otherwise mentioned, selective cultures and sub-cultures were prepared in 50 ml serum vials sealed with butyl rubber stops and aluminum crimps. As a final step, after inoculum addition and sealing of the vials, the headspace of the estuary sediments cultures was replaced by a gas mixture of N_2:O_2 (85:15) and N_2 in the deep-sea selective cultures. Vials were kept in inverted position during the incubation in the dark at 25 °C[98]. Abiotic controls for all conditions were also included. The detailed procedures are described in the following sections.

4.4.2.1. Selective cultures of estuarine sediments

The mineral salts medium (MSM) was adapted from Domingues *et al*. (2013)[205]. The main solution contained NaCl 17.0 g/L, $MgCl_2 \cdot 6H_2O$ 5.8 g/L, Na_2HPO_4 3.0 g/L, $NaHCO_3$ 2.5 g/L, KH_2PO_4 2.0 g/L, NH_4Cl 0.7 g/L, KCl 0.7 g/L, cycloheximide 0.1 g/L, resazurin 1.0 mg/L, $FeCl_3 \cdot 6H_2O$ 0.5 mg/L, $ZnCl_2$ 5.0 x 10^{-2} mg/L, $CaCl_2$ 2.0 x 10^{-2} mg/L, $CuSO_4$ 5.0 x 10^{-3} mg/L and $MnCl_2 \cdot 4H_2O$ 5.0 x 10^{-3} mg/L. The pH was adjusted to 7.2 ± 0.2. To select for metabolically diverse hydrocarbonoclastic anaerobic bacteria, different TEAs and chemical reducing agents (Table 4.7) were used in the selective cultures with different sediment types (Table 4.8). Flasks were filled with medium to 4:5 of the total volume for fermentative conditions and 9:10 for sulfate-reducing bacteria (SRB) and NRB conditions. Initial cultures were inoculated with 10 % (w/v) of the corresponding

Table 4.7 – Supplements to MSM (g/L medium).

Supplements	Fermentative	SRB	NRB	Solid MSM
Na_2SO_4	-	2.00	-	2.00
KNO_3	-	-	2.00	2.00
$Na_2S \cdot 9\ H_2O$	0.24	0.24	-	-
Cysteine-HCl	0.47	0.47	-	-
Ascorbic acid	-	-	0.71	0.71
Agarose	-	-	-	20.00

sediments and 1 % (v/v) of crude oil was added to 200 ml flasks. The cultures were incubated for 5 weeks after which an aliquot of 10 % (v/v) was transferred to fresh medium in 50 ml vials. Three more transfers to fresh medium were conducted in the same way so that overall incubation period of the selective cultures was 20 weeks.

Table 4.8 – Designation of the selective cultures of estuarine sediments in relation to sediment type and selective pressure for certain anaerobic metabolic types.

		Metabolic selective pressure		
		NRB	*SRB*	*Fermentative*
Inocula	Abiotic control	Ø -NRB	Ø -SRB	Ø -Fer
	Muddy	M-NRB	M-SRB	M-Fer
	Sandy	S-NRB	S-SRB	S-Fert

4.4.2.2. Selective cultures of deep-sea sediments

For the preparation of selective cultures of hydrocarbonoclastic SRB and NRB from deep-sea MVs sediments, full marine medium was used as described in Widdel (2010), with some adaptations (Table 4.9)[98]. Resazurin 0.001 g/L was added to the main solution, the vitamin mixture and thiamine solution were replaced by RPMI 1640 Vitamins Solution 100X (Sigma) 10 mL/L, KNO_3 was used instead of $NaNO_3$ and cycloheximide 0.01 g/L was added to the final medium.

With the aim of assessing the effects of nitrate, sulfate and crude oil amendment on bacterial communities of active and inactive MVs, a complex experimental design was prepared involving 56 serum vials per incubation period, including three cultures per condition prepared with three

Table 4.9 – Supplements to media used in cultures with deep-sea sediments according to the different selective factors (g/L $_{medium}$ except when mentioned otherwise).

	Selective pressure							
	No supplement	*NO_3^-*	*SO_4^{-2}*	*NO_3^- + SO_4^{-2}*	*Crude oil*	*NO_3^- + crude oil*	*SO_4^{-2} + crude oil*	*NO_3^- + SO_4^{-2} + crude oil*
Na_2SO_4 [(a)]	0.50	0.50	4.00	4.00	0.50	0.50	4.00	4.00
$Na_2S \cdot 9 H_2O$	-	-	0.24	0.24	-	-	0.24	0.24
KNO_3	-	1.00	-	1.00	-	1.00	-	1.00
Inoculum (mL/L)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Crude oil (m/L)	-	-	-	-	44.40	44.40	44.40	44.40

[(a)] Present in main solution.

sediment sub-samples (identified as a, b and c) from the same corer, and abiotic controls (Table 4.10). Serum vials were filled up to 9:10 capacity with medium prepared according to the selective factors. In cultures with light Arabian crude oil (C and NC), 1% (v/v) of the carbon source was added, and in cultures without amendment or with only crude (0 and C) KNO_3 was absent. For the initial cultures, 50 mg of soil was added to 45 mL culture medium, and in the two subsequent transfers to fresh medium, 10% (v/v) of the previous culture was used as inoculum. Incubation in fresh media lasted 5 weeks, totaling an incubation period of 15 weeks.

4.4.3. Detection of biological reduction of nitrate and sulfide production

After each incubation period of the selective cultures with estuarine sediments and after the final period of incubation of MV sediment cultures, biochemical assays were performed under aerobic conditions to assess the activity of SRB or NRB.

The activity of NRB was detected by the nitrate reductase test. Test tubes containing nitrate medium (tryptone, 20.0 g/L; disodium phosphate, 2.0 g/L; dextrose, 1.0 g/L; potassium nitrate, 1.0 g/L; agar, 1.0 g/L; pH 7.2 ± 0.2)[485], were inoculated with 50 µL of each selective culture, and covered with a 0.5 cm layer of liquid paraffin. Cultures were incubated at 25 °C for two days. After incubation, a few drops of 0.8% 4-aminobenzenesulfonic acid and 0.5% n,n-dimethyl-1-naphthylamine were added to each tube. The appearance of a red color, indicating the presence of nitrite, was interpreted as positive result. If no color change was observed, a small amount of zinc powder was added to reduce the remaining nitrate to nitrite. Subsequent change to red color, confirming that nitrate has not been biologically reduced, was considered a negative result and no

Table 4.10 – Designation of the selective cultures of deep-sea sediments in relation to inoculum and selective pressure for anaerobic metabolic types. Whenever sub-sample identification is required the letters a, b and c are used for the different sub-samples.

		Selective pressure							
		No supplement	*NO_3^-*	*SO_4^{-2}*	*NO_3^- + SO_4^{-2}*	*Crude oil*	*NO_3^- + crude oil*	*SO_4^{-2} + crude oil*	*NO_3^- + SO_4^{-2} + crude oil*
Inocula	Abiotic control	Ø-0	Ø-N	Ø-S	Ø-NS	Ø-C	Ø-NC	Ø-SC	Ø-NSC
	Active MV	A-0	A-N	A-S	A-NS	A-C	A-NC	A-SC	A-NSC
	Inactive MV	I-0	I-N	I-S	I-NS	I-C	I-NC	I-SC	I-NSC

color change a positive result, indicating that nitrate was no longer present and that nitrite had been further reduced to N_2 or ammonia[486].

Growth on Triple Sugar Iron (TSI, Liofilchem) medium was used as an indicator of possible SRB in the selective cultures. The medium was dispensed in test tubes and inoculated with a needle up to a 3 cm depth. The cultures were covered with a 0.5 cm layer of liquid paraffin (Merck) and incubated at 25 °C for 2 days (estuarine) or 3 months (deep-sea). Positive results were indicated by the appearance of a dark precipitate, resulting from the formation of iron sulfide due to utilization of the thiosulfate anion as a TEA[486]. While using the thiosulfate anion as a TEA is not a metabolic process exclusive of SRB due to the nature of the inoculum it is expected to be a fair indicator of the presence of SRB in the selective cultures.

4.4.4. Isolation and purification of bacterial strains

After each incubation period of 5 weeks, aliquots of 200 µL of each culture were spread-plated on plates of solid MSM medium to which a superficial layer of 50 µL of crude oil was previously added. The plates were incubated under aerobic conditions, in the dark, at approximately 25 °C for at least one month. Randomly selected isolates were purified by three consecutively streak-plating in identical medium and incubating in similar conditions. Isolates were then inoculated in test tubes with 10 mL half-strength TSB and 1 mL crude oil. Aliquots of 1.5 mL of the isolates cultures were frozen with glycerol (20 %; AppliChem) and kept at -80 °C until processing.

4.4.5. DNA extraction of bacterial isolates

Aliquots of glycerol-amended frozen cultures were inoculated in 10 mL of Marine Broth 2216 (MB2216; Difco) in test tubes with 100 µL crude oil. The cultures were incubated for a month under aerobic conditions at 25 °C. DNA extraction was performed following the protocol by Henriques *et al*. (2004)[487]. The final DNA–RNA pellet was resuspended in 50 µL of TE buffer and stored at −80 °C.

4.4.6. Identification of isolates

BOX-PCR was conducted in order to identify isolates with similar genotypes and to group them in clones according to their similarity, and to select clone representatives for identification thus reducing the DNA sequencing effort. The composition of the reaction mixture (25 µL) was 1 µL of sample, 12.5 µL DreamTaq™ PCR Master Mix (Thermo Fisher Scientific), 0.50 µL of primer, 1.25 µL dimethyl sulfoxide, and 9.75 µL dH_2O. The PCR cycle was 7 min at 94 °C, followed by 35 thermal cycles of 1 min at 94 °C, 2 min at 53 °C, and 8 min at 65 °C. A final extension step at 72 °C for 16 min was performed. The primer used was the BOX A1R[488]. The PCR products were stored at

-20 °C until analysis by gel electrophoresis in agarose gel (1.5 %) containing 5.3 x 10^{-3} % (v/v) RedSafe, immersed in TAE buffer 1x, at 80 V for 3h. The profiles were visualized using a ChemiDoc XRS+ System scanner and Image Lab software (BioRad). The profiles obtained were analyzed with BioNumerics v6.6 (Applied Maths, Belgium) and Primer5 software.

The 16S rRNA gene of each BSF-producing bacterial isolate presenting distinct BOX A1R profiles was PCR amplified using the universal bacterial primers 27F and 1492R[375]. The composition of the reaction mixture (25 µL) was 1 µL of sample, 12.5 µL DreamTaq™ PCR Master Mix, 0.25 µL of each primer, 1 µL BSA (2 g/L; Sigma), and 10 µL dH_2O. The PCR cycle was as described in Domingues *et al*. (2013)[205]. The success of the amplification of the 16S rRNA gene fragments was verified by agarose gel (1 %) electrophoresis, with 5.3 x 10^{-3} % (v/v) RedSafe (Intron Biotechnology) as DNA staining agent. The amplicons were sequenced by StabVida (Portugal). The obtained sequences were matched to the sequences available in the GenBank database using BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm. Nih.gov) in order to determine their closest relative.

4.4.7. Analysis of BSF production

Before and after identification, isolates were tested for BSF production by the atomized oil assay[489]. After inoculation in Luria Agar (LA; Liofilchem) or MB2216 (supplemented with 2% agarose) with sterile needle, cultures were incubated under aerobic conditions at 25 °C until small colonies were visible. Identical anaerobic cultures were incubated in an anaerobic jar, with the reagent Anaerocult® A (Merck) and indicator Anaerotest® (Merck), at 25 °C in the dark for a minimum of 5 weeks. Pulverization of the plates with liquid paraffin using an airbrush (model BD-128P, Fengda, China) allowed the detection of halos characteristic of positive results for the production of BSFs. Chemical surfactant solution, SDS 25 %, and *Pseudomonas* sp. 74[205] were used as positive controls and *Escherichia coli* DH5α as a negative control.

Identified isolates were grown in 100 mL cultures of Luria Broth (LB; Liofilchem) supplemented with NaCl for a total of 20 g/L or MB2216, with 1% crude oil. Incubation took 1 week in aerobic conditions after which 1 mL was transferred to fresh medium and incubated for 3 weeks under anaerobic conditions in serum vials. For anaerobic media, sodium ascorbate (0.0015 M) was used as an oxygen scavenger[98]. Incubation was conducted at 25 °C, in the dark, with orbital shaking of 100 rpm. After aerobic and anaerobic incubation periods, 50 mL of each culture was centrifuged at 16,000 g for 5 minutes and the supernatant was filtered using 0.2 µm pore size membranes to remove bacterial cells[490]. The filtered supernatant was used for the drop collapse test and for surface tension and emulsification index (E_{24}) measurements. An adapted drop collapse test[491] was performed in new plastic petri dishes in which aliquots of 1 µL of either filtered supernatant, SDS 25 % as

positive control or dH_2O, LB and MB2216 as negative controls, were added to 5 µL droplets of dH_2O pipetted onto the surface. After one minute the drop diameter was measured with an ocular micrometer adapted to a binocular stereoscopic microscope. Surface tension measurements of the isolate cultures filtered supernatant were performed by a surface tensiometer DST9005 (Nima Technology, UK) with a DuNuöy ring at room temperature[492,493]. The E_{24} was measured after 24 hours from emulsification as described in Pereira *et al*. (2013)[53]. Instead of *n*-hexadecane liquid paraffin (Merck) was used.

Hemolytic activity can be an indicator of BSF production by the formation of halos surrounding the colonies. Isolates were grown in Columbia Blood Agar Pharm. Plates (Merck) in the anaerobic jar under the conditions mentioned above for the anaerobic atomized oil assay[494].

Statistical analysis was performed in GraphPad Prism 6. BSF production results obtained through the drop collapse method, the E_{24} and surface tension measurements were compared with the corresponding abiotic media by an unvaried analysis of variance (ANOVA) model with the Tuckey post hoc test. A value of $p < 0.05$ was considered significant.

4.5. Results and discussion

4.5.1. Selective cultures

Aiming at selecting hydrocarbonoclastic facultative anaerobes from estuarine and deep-sea sediments, anaerobic selective cultures were prepared using only crude oil as carbon source and subaquatic sediments as inoculum. The media used in the selective cultures varied in accordance to the anaerobic respiration metabolism for which we were selecting, SRB, NRB or fermentative bacteria in the absence of TEAs. To confirm the effect of selective pressure in the cultures, biochemical assays for NRB and possible SRB bacteria were used. Since using the thiosulfate anion, which is found in the TSI medium, as a TEA resulting in the production of sulfide is not a metabolic process exclusive of SRB, this test is used only as an indicator of the presence of possible SRB.

4.5.1.1. Estuarine sediments

In cultures with estuarine sediments NRB and possible SRB bacteria were present in sandy and muddy sediments, although with different frequency. TSI-positive results (Table 4.11), were observed at the end of the first incubation periods, in all selective media. Sulfide production was also detected in NRB cultures prepared with muddy or sandy sediments. In SRB selective cultures, sulfide production was only detected with muddy sediment inoculum. In fermentative conditions, sulfide production was only detected at the end of the first incubation period, in the culture prepared with sandy sediment. Sulfide formation was not observed after the second incubation period, in any of the

cultures. NRB were detected in all NRB-selective cultures but were also detected in the initial incubation periods in the SRB and fermentative selective cultures (Table 4.12). This cross-selection for NRB in sulfate-reducing and fermentative media may indicate the presence of bacteria able to use different TEAs[495] and/or that NRB are more tolerant to oxygen limitation and nutrient scarcity than SRB and fermentative bacteria. It should be noticed that many of the negative results, especially those obtained after period IV, most likely corresponded to the absence of growth. NRB appear to be more predominant than sulfide producers in all selective cultures, especially in those with the NRB-medium. In NRB-selective conditions, nitrate reduction was detected in cultures with both types of sediment throughout the whole incubation period. In other selective media, nitrate reduction was no longer detected in the last five weeks incubation period. These results indicate that selective pressure in NRB-medium cultures successfully led to an enrichment of NRB. Media conditions and the duration of the experiment affected the prevalence of NRB and sulfide-producing bacteria in the selective cultures. Of both groups of bacteria selected from estuarine sediments and tested for, NRB appears to be the most likely to be isolated, cultured and maintained especially in media with nitrate. It is possible this is because many NRB are facultative anaerobes[253], while most SRB are strict anaerobes[129], and therefore may be less tolerant to the aerobic conditions in which the biochemical tests were performed.

Table 4.11 – Results of biochemical assay using TSI medium for testing the production of sulfide by bacteria at the end of the four incubation periods during a five-week experiment in selective cultures with estuarine sediments. + indicates a positive result, - indicates a negative result and * indicates that no growth was observed.

Incubation period	Soil type	Selective cultures		
		NRB	*SRB*	*Fermentative*
I	Sand	+	-	+
	Mud	+	+	-
II	Sand	+	-*	-*
	Mud	+	+	-*
III	Sand	-*	-*	-*
	Mud	-*	-	-*
IV	Sand	-*	-*	-*
	Mud	-*	-*	-*

Table 4.12 – Results of the nitrate reduction test at the end of the four incubation periods during a five-week experiment in selective cultures with estuarine sediments. + indicates a positive result, - a negative result and * that no growth was observed.

Incubation period	Soil type	Selective cultures		
		NRB	*SRB*	*Fermentative*
I	Sand	+	+	+
	Mud	+	+	+
II	Sand	+	-	+
	Mud	+	+	+
III	Sand	+	+	+
	Mud	+	-*	-
IV	Sand	+	-	-
	Mud	+	-	-

4.5.1.2. Deep-sea sediments

Samples from deep-sea sediment obtained from an active and an inactive MV in the Gulf of Cadiz were also used in the preparation of selective cultures which aimed at selecting hydrocarbonoclastic bacteria and other specialized groups, such as BSF-producing bacteria, which could improve hydrocarbon degradation. Overall, 56 selective cultures, including abiotic controls and three sediment sub-samples, were prepared varying in the presence or absence of crude oil, as sole carbon source, nitrate and/or sulfate, as possible TEAs.

Preliminary biochemical assays were used after the final incubation period to confirm the selection of NRB and SRB bacteria. Sulfide production test was negative for all conditions and inoculum. However, since the test was not performed under strict anaerobic conditions, it is possible that bacteria sensitive to O_2 that were present in the selective cultures may have been inactivated under the test conditions. Nitrate reduction was observed in selective cultures of the two MV sites and at least once under all conditions (A-0, A-N, A-S, A-NC, I-S, I-C and I-CN), except in cultures with nitrate and sulfate (NS), sulfate with crude (SC) and nitrate, sulfate and crude (NSC). Some results were inconclusive, due to contamination by fungi, probably because of the long incubation (3 months). However, the overall results indicate that both active and inactive MV sediments were sources of NRB. The lack of evidence of nitrate reduction in selective cultures where nitrate and sulfate were both present, with or without crude oil, may be interpreted as an indication that SRB or bacteria able to use other TEAs outcompete NRB under these conditions or that redox conditions

were too severe for NRB, since sodium sulfide was used as reducing agent. These results agree with the results of the pyrosequencing of the 16S rRNA gene fragments of the communities, in which genera containing known nitrate reducers were detected, such as, *Planomicrobium* spp. (in cultures Aa-C, Ab-NC and Aa-NC) and *Variovorax* spp. (present in most selective cultures, independently of sediment origin or condition), have been identified (Chapter 3).

Production of BSF by the bacterial communities was also tested under aerobic conditions, using the atomized oil assay. Production was detected in at least one replicate of each condition and MV, except for A-0 and A-NSC. It is possible that in these two cases only non-BSF producing bacteria grew on the plates, outcompeting BSF-producers. This indicates the high potential of MV sediments as potential sources of BSF-producing oxygen-tolerant anaerobes.

4.5.2. Isolation and identification of isolates

After each incubation period an aliquot of each selective culture was plated under aerobic conditions in MSM with only crude oil as carbon source. From these plates, 37 isolates were retrieved from selective cultures of estuarine sediment and 23 from the selective cultures of deep-sea sediments. BOX-PCR was used to screen for redundant isolates (> 90% similarity). After molecular typing, 24 isolates from the estuarine sediments and 11 isolates from the deep-sea sediments were selected for further identification and characterization. Most (14) of the estuarine isolates were obtained from the cultures selecting for NRB, five from the fermentative conditions and five from the SRB cultures. The sediment texture did not appear to affect the isolation of bacteria from the estuarine sediments. On the other hand, on deep-sea cultures almost all of the isolates, except for two, were isolated from cultures inoculated with sediment from an inactive MV. However, the isolates came from selective cultures submitted to all types of amendments.

Six and seven isolates from the estuarine and the deep-sea sediments, respectively, were detected as being able to produce BSFs under aerobic conditions using the atomized oil assay. This confirms previous reports regarding the estuarine system of Ria de Aveiro[205,496] and the deep-sea[231,497] as a sources for hydrocarbonoclastic and BSF-producing bacteria. These isolates were identified by sequencing of 16S rRNA gene fragments (Table 4.13). All the deep-sea isolates identified are represented in the classification of OTUs from the pyrosequencing results (Chapter 3). The most common taxon was *Pseudomonas* (DS27, DS192, R47 and R53), followed by *Bacillus* (DS61 and DS230) and *Ochrobactrum* (R98 and R114). Both *Pseudomonas* and *Bacillus* are two of the genera

Table 4.13 – BLAST results obtained for the 16S rRNA gene sequence alignments of the 13 BSF-producing isolates. DS indicates isolates from the deep-sea selective cultures and R from the estuarine selective cultures.

Isolate No.	Selective Culture	Sequence Accession No.	Closest relative		
			BLAST-N Identity	Accession No.	% Identity
DS27	Ab-0	MF490026	*Pseudomonas sp.*	KR088605	99
DS61	Ia-N	MF490027	*Bacillus subtilis*	KU862331	100
DS72	Ic-S	MF490028	*Brevundimonas sp.*	LN833256	100
DS104	Ib-NS	MF490029	*Psychrobacter sp.*	KF859544	100
DS140	Ic-C	MF490030	*Staphylococcus sp.*	KT282233	99
DS192	Ic-NC	MF490031	*Pseudomonas sp.*	KX301316	100
DS230	Ic-SC	MF490032	*Bacillus sp.*	MF398308	99
R21	S-Fer	MF490033	*Marinobacter salsuginis*	KM041132	99
R33	S-SRB	MF490034	*Curtobacterium flaccumfaciens*	KF003415	100
R47	M-SRB	MF490035	*Pseudomonas xanthomarina*	KY465430	99
R53	M-NRB	MF490036	*Pseudomonas sp.*	LN877946	99
R98	M-NRB	MF490037	*Ochrobactrum sp.*	KJ676969	100
R114	S-SRB	MF490038	*Ochrobactrum sp.*	JQ014582	100

in which aerobic and anaerobic BSF production is best represented, including many PH-degrading species[475]. Members of *Ochrobactrum* are known as hydrocarbonoclastic BSF-producers, degrading mainly polycyclic aromatic hydrocarbons (PAHs)[498,499]. They are also known as NRB[500] or able to ferment organic compounds[501] under anaerobic conditions. However, anaerobic production of BSF has not been reported before. Other isolates belong to the *Brevundimonas, Psychrobacter, Staphylococcus, Marinobacter* and *Curtobacterium* genera. All these genera include facultative anaerobes[502–504]. Members of *Curtobacterium* and *Brevundimonas*, in particular, are known for versatile anaerobic metabolism, being able to use several TEAs such as nitrate, iron[505] or arsenic[506].

Brevundimonas, Staphylococcus, Psychrobacter and *Marinobacter* all contain PHs degrading species. The first two are often associated with PAH degradation[507,508], *Psychrobacter* is known to degrade alkanes[509] and *Marinobacter* has been reported to degrade both types of PHs[504]. BSF production under aerobic conditions has also been observed in these four genera[510–513]. However, BSF production under anaerobiosis has not been reported for the first three, and while a *Marinobacter* member has been associated with anaerobic BSF production by a consortia it is not clear if it was directly responsible for the production[514]. *Curtobacterium* genus is still poorly studied. So, no member has been directly associated with aerobic or anaerobic BSF production or PHs degradation despite having been found in PHs contaminated sediments[515]. All genera have previously been found in subaquatic sediments[427,498,512,516–519]. *Pseudomonas* and *Ochrobactrum* have been described in previous studies regarding PH degrading bacteria from the estuary system of Ria de Aveiro[205,520]. *Pseudomonas* are in fact the best represented genus in the rhizosphere, roots, sediment and water of the sampling site in the estuary of Ria de Aveiro[205,496,520]. While in this study only two *Pseudomonas* were isolated from estuarine sediments, it is possible that the anaerobic selective cultures pressured the sediments communities to adapt to the lack of oxygen favoring the growth of facultative anaerobes instead of obligate aerobes. Thus explaining the *Marinobacter* and *Curtobacterium* isolates which had not yet been identified in this estuarine system.

The fact that isolates retrieved from selective cultures of MV sediments are associated with degradation of PAHs is consistent with previous findings in which communities from inactive MVs presented a preference for degradation of PAHs instead of simpler PHs such as alkanes and monoaromatic hydrocarbons (Chapter 3).

4.5.3. Biosurfactant production

4.5.3.1. Growth on different culture media

MSM medium was used for isolation of bacteria and their purification. However, since the MSM medium is a complex medium requiring laborious preparation other commercially available and simpler to prepare formulas were tested as growth media to be used in further BSF production tests. LA is a simple medium know to grow a wide variety of bacteria, such as BSF producing bacteria previously isolated from the Ria the Aveiro estuary[205], and is also used in the description of the atomized oil assay method[489]. MB2216 is a highly complex factory prepared media designed to mimic the chemical composition of marine environments, and as such it was expected to meet the nutritional demands of most, if not all, isolates. Since the salinity of LA is low compared to the salinity of MB2216, NaCl concentration was adjusted to 20 g/L. All estuary isolates were able to grow in solid MB2216 as well as in LA. However deep-sea isolates DS27, DS72 and DS230 only

grew in solid MB2216. Other deep-sea isolates, DS140 and DS61, presented faster growth in in solid MB2216. Since MB2216 medium is more complex in terms of chemical composition and more specific for marine bacteria, it is possible that it contains some trace nutrients fundamental for the growth of deep-sea bacteria that are lacking in LA. In further tests, LA medium was used to test BSF aerobic and anaerobic production ability in all the estuarine isolates (Table 4.14) and also for two deep-sea isolates (DS192 and DS104). MB2216 was used for all other deep-sea isolates. For the atomized oil assay both media were used.

Table 4.14 – Characterization of BSF production indicators by identified isolates. * Surface tension reduction between the supernatant of each isolate's culture and the corresponding abiotic control. E_{24}: emulsification index. +: positive result, - negative result. NA: not available.

Isolates	**Surface tension reduction* (mN/m)**		**E_{24} (%)**		**Atomized oil assay**			
	Aerobic	*Anaerobic*	*Aerobic*	*Anaerobic*	*LA – Aerobic*	*LA – Anaerobic*	*MB2216 – Aerobic*	*MB2216 – Anaerobic*
DS27	5.10 ±2.54	0.11 ±0.70	0.0 ±0.0	0.0 ±0.0	+	-	+	-
DS61	2.70 ±0.36	0.16 ±0.40	2.4 ±2.1	7.8 ±6.3	+	-	+	+
DS72	2.78 ±1.55	4.32 ±3.48	0.0 ±0.0	6.5 ±2.4	-	+	+	+
DS104	5.05 ±1.02	-0.65 ±1.10	55.9 ±7.5	5.1 ±1.9	+	+	+	+
DS140	-0.73 ±1.00	0.05 ±0.51	6.5 ±0.8	1.7 ±1.4	+	+	+	+
DS192	0.32 ±0.28	-1.98 ±3.01	8.2 ±1.3	15.9 ±1.2	+	+	+	+
DS230	5.26 ±0.28	0.06 ±3.48	0.0 ±0.0	0.0 ±0.0	NA	NA	NA	NA
R21	1.95 ±3.23	0.35 ±0.68	4.3 ±4.2	7.5 ±3.0	NA	NA	NA	NA
R33	5.93 ±0.22	0.68 ±0.42	57.7 ±6.8	8.2 ±3.1	+	-	+	+
R47	10.01 ±0.13	5.23 ±4.20	64.0 ±1.8	16.7 ±14.5	+	+	+	-
R53	6.68 ±0.38	2.42 ±0.53	46.7 ±5.9	35.0 ±9.3	-	+	+	+
R98	8.10 ±0.35	1.64 ±0.71	12.7 ±7.9	8.1 ±4.9	+	-	+	+
R114	9.76 ±0.87	0.16 ±0.50	12.4 ±6.4	19.8 ±9.5	+	+	+	+

4.5.3.2. Different methods for detection of biosurfactant production

The capacity to produce BSF could not be demonstrated for all isolates from a sole methodological approach. BSF production was not detected for any isolate by hemolysis in blood agar nor the drop collapse tests. The E_{24} identifies the presence of emulsifiers, which is often, although not always, is a property of BSFs[521]. As such, the results of this method must be complemented with other evidence of BSF production, such as the atomized oil assay (Figure 4.14) or surface tension measurements. In some cases (e.g. DS230), halos were not detected in the atomized oil assay despite a decrease of surface tension and the opposite was also observed (e.g. DS140). It is possible that BSF concentration in the culture supernatants was too low for a detectable decrease of surface tension, which is also the most probable reason for why all isolates presented negative results in drop collapse tests, which is known to require relatively high concentrations of BSFs for positive results[489]. The atomized oil assay is known to be much more sensitive method than the drop collapse[489]. The isolates that presented negative results in the atomized oil assay are the same that were difficult to maintain in the cultures after successive inoculations (R21 and DS230). Both R21 and DS230 isolates appear to have lost its BSF production ability in solid media with successive inoculations. Positive results were observed in the atomized oil assay used in preliminary tests with LA to identify possible BSF producers, but growth was not observed in latter tests in any of the media. R21 gave positive results for the atomized oil assay under aerobiosis and anaerobiosis (Figure 4.14), while DS230 only tested positive in aerobiosis. In latter incubations for atomized oil assay no growth was observed. It is possible that overall the conditions used were not very amenable for growth. In tests involving liquid cultures of R21 some emulsification was observed under both

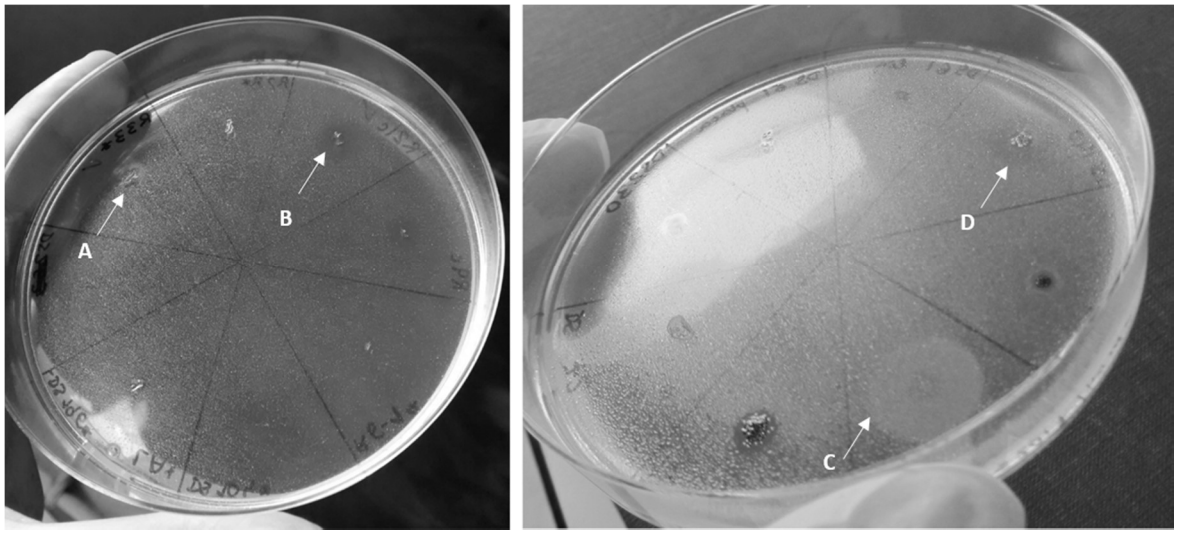


Figure 4.14 – Photographs depicting examples of results of the atomized oil assay for plates incubated in the anaerobic jar. On the left, an LA plate with a bright halo for R33 (A) and a dark halo for R21, both positive results. On the right, a MB2216 plate with a bright halo for the positive control of SDS 25% (C) and a dark halo for DS140.

aerobic and anaerobic condition, although a significant effect on surface tension was not (Table 4.14). In DS230, a significant decrease of surface tension was observed only in aerobiosis, with no indication of BSF being produced by this isolate under anaerobiosis.

Decrease of surface tension is an effect of the presence of BSF in solutions. The decrease is related to BSF concentration, decreasing very little after the critical micelle concentration is reached[522]. The supernatant of aerobic cultures of isolates DS230, R47, R98 and R114 caused significant reduction of the surface in comparison to the abiotic controls. No significant surface tension decrease was observed under anaerobic conditions. Since all isolates are known BSF producers it is possible that BSF concentrations were not high enough for a difference in surface tension to be detected. In future studies BSFs should be extracted for further characterization, including determination of critical micelle concentration. So that the lowest possible decrease of surface tension can be determined.

The E_{24} indicates the presence of molecules that promote stable emulsions for 24 h[521]. Significant emulsification was observed in aerobic cultures of R33, R47, R53 and DS104, and in anaerobic cultures of R53. All significant results were obtained with LB cultures. As previously mentioned, both LB and MB2216 are very similar in macronutrient composition and therefore it is possible that some of the mineral salts of MB2216 affected BSF structure leading to a decrease in its emulsifying ability.

4.5.3.3. Aerobiosis vs. *anaerobiosis*

Overall, BSF production increased under aerobic conditions in relation to anaerobiosis. In most isolates, the supernatant of aerobic cultures caused a greater reduction in surface tension (e.g. DS230 and R47). Higher BSF production in aerobiosis is to be expected[475] since anaerobic metabolism is slower and energetically less favorable than aerobic metabolism[95]. On the other hand, the percentage of BSFs isoforms produced can be affected by the oxygen concentration during growth, leading to BSFs produced by the same bacteria but with different characteristics[56,86]. This could explain why the supernatant of anaerobic cultures of isolate R114 presented higher E_{24}, even though less BSF was produced, as inferred from the surface tension test, in comparison. As such, further testing is required to characterize the BSFs produced under both aerobic and anaerobic conditions, since a particular structure may have more desirable properties than other that is produced with higher yield.

4.5.3.4. Effect of culture medium in biosurfactant production

Optimization of culture media may also improve anaerobic BSF production and minimize the effect of lack of oxygen on BSF yields[78]. In some cases it was noted that the same bacteria grown on different media produced different sized halos in the atomized oil assay under the same oxygen conditions. This effect was probably related to the amount of BSF produced, showing that the composition of the culture medium affected BSF production. Furthermore, in LA, BSF production by DS72 and R53 was only detected under anaerobic conditions, and BSF was only produced by DS61, R33 and R98 under aerobic conditions. In MB2216 production of BSF for these isolates was observed under both aerobic and anaerobic conditions. As such, the choice of medium used in the atomized oil assay could in the extreme lead to false negatives, especially considering that LA is one of the media used in the original description of the method[489]. These results support the fore mentioned findings that bacterial growth is generally improved in MB2216 when compared to LB. Since MB2216 is a more complex media than LA it is possible that some nutrients present in the first lead to better cell growth and in turn BSF production. For R47, BSF production under anaerobic conditions was only observed in LA. Considering that LA have been adjusted to a similar NaCl concentration than MB2216 (20 g/L), and both media use peptone and yeast extract as carbon sources, the fact that some bacteria prefer LA can only be attributed to an inhibiting influence by some of the marine salts present on MB2216.

4.5.3.5. Most promising isolates

Overall, the most promising isolates for production of BSF in aerobic conditions were retrieved from selective cultures of estuarine sediments. R33 presents the second highest E_{24} (57.65 ±6.79 %). R53 is the only isolate to cause significant emulsification in the presence (46.67 ±5.90 %) and absence (34.97 ±9.31 %) of oxygen, indicating the production of a BSF with strong emulsifying properties. R114 and R98 are also associated with significant decreases of surface tension (8.10 ±0.35 mN/m and 9.76 ±0.87 mN/m), respectively, and both produced wide halos in the atomized oil assay.

R47 is a promising isolate for aerobic and anaerobic BSF production. It caused the greatest decrease of surface tension both in aerobic (10.01 ±0.13 mN/m) and anaerobic cultures (5.23 ±4.20 mN/m), as well as the highest E_{24} (63.96 ±1.75 %) for aerobic cultures.

Some of the most promising isolates from the deep-sea are DS61, DS104 and DS140, which produced some of the widest halos in aerobic as well as in anaerobic conditions. DS104 is the only deep-sea isolate with significant E_{24} determined for aerobic cultures.

Some of the best BSF producers belong to genera for which BSF production had not yet been reported. For isolate R33 identified as a *Curtobacterium* species, BSF production was for the first

time detected in aerobic and in anaerobic cultures. For isolates R98, R114, DS104 and DS140, which have been classified as *Ochrobactrum* (the first two), *Psychrobacter* and *Staphylococcus*, this the first report of BSF under oxygen limitation. This is also true for less productive isolates such as DS72 and R21, identified as *Brevundimonas* and *Marinobacter*, respectively. This is probably due to the fact that most bacteria are not tested for anaerobic BSF production due to material and time constrains[475].

Pseudomonas sp. 74, a BSF-producing bacteria previously isolated from the rhizosphere of halophytes in the estuary of Ria de Aveiro[205] was used as positive control of the aerobic atomized oil assay, presenting positive results in both solid MB2216 and LA. This isolate was also tested under anaerobiosis and BSF production was also observed in this condition. As such, it is also a promising candidate to further studies to detail anaerobic BSF production.

The isolates described in this work represent a high biotechnological potential for industrial BSF production, bioremediation and MEOR strategies, especially under anaerobic conditions or, as aerotolerant, in environments that oscillate between aerobiosis and anaerobiosis. Future work should involve characterization of the produced BSF and optimization BSF production.

4.6. Conclusion

Thirteen isolates belonging to *Pseudomonas*, *Bacillus*, *Ochrobactrum*, *Brevundimonas, Psychrobacter, Staphylococcus, Marinobacter* and *Curtobacterium* genera have been isolated from selective cultures prepared with deep-sea and estuarine subaquatic sediments. All isolates were able to grow with crude oil as sole carbon source and all produced BSF under aerobic and anaerobic conditions, with the exception of DS27 and DS230 which only did so under aerobiosis. Subaquatic sediments of marine environments have proven to have a high potential for isolation of aerobic and anaerobic hydrocarbonoclastic and BSF-producing bacteria. These environments are still fairly unknown which could lead to the discovery of new strains with biotechnological potential and, consequently, of novel BSFs.

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Chapter 5

CHAPTER 5

Conclusion and future perspectives

The review of the state-of-the-art on biosurfactant (BSF)-production in microaerobic and anaerobic conditions revealed that so far only a few bacterial strains or consortia are known to produce BSFs under oxygen-limiting conditions. Most of these strains belong to either *Bacillus* or *Pseudomonas*. The reason why there are not more known bacteria with this ability is due to lack of research under conditions lacking oxygen, which is often more time consuming and requires specific material than research under aerobic conditions. Furthermore, it was shown that culture media optimization can affect the composition of BSFs produced, leading to different BSF characteristics, as well as, increased BSF concentrations. This is also observed for hydrocarbonoclastic bacteria, which are wildly used in aerobic microbial enhanced oil recovery (MEOR) and bioremediation strategies but few approaches are proposed for the application of these strategies under oxygen-limiting conditions. This is of particular interest considering that many petroleum reservoirs requiring MEOR present anaerobic or microaerobic conditions, and petroleum hydrocarbons (PHs) contamination can occur in environments lacking oxygen, such as what happened in the Deepwater Horizon oil spill in 2010.

This work was aimed at increasing the current knowledge regarding hydrocarbonoclastic BSF-producing bacteria with biotechnological potential, particularly under oxygen-limiting conditions. This was achieved through two approaches: preparation of anaerobic enrichment cultures, with or without crude oil and/or nitrate, that were used to assess the bacterial communities involved in hydrocarbon degradation in deep-sea mud volcanos (MVs); while selective media were used to isolate and characterize bacterial strains with biotechnological potential from deep-sea MVs sediments and subtidal estuarine sediments from Ria de Aveiro.

The first approach allowed an in-depth analysis of anaerobic bacterial communities of sediments from deep-sea MV with different types of activity and their responses to the presence or absence of nitrate and/or crude oil. A major tendency was the differentiation of communities due to the MV activity, independently of experimental conditions. Overall, Alphaproteobacteria and Bacilli, predominated in cultures of active and inactive MVs, respectively. However, smaller taxonomic units responded to the enrichment factors. In active MV cultures, crude oil led to the appearance of members of Planococcaceae family, but the presence of nitrate together with crude oil resulted in a decrease in their relative abundances. Crude oil also increased the relative abundances of Erythrobacteraceae and the decrease of Actinobacteria. In inactive MV cultures, crude oil positively affected Bacillaceae, Acidimicrobiales and Flavobacteriaceae. Nitrate appear to have favored

Nocardioidaceae in some active MV cultures and Bacillaceae in inactive MV cultures. Analysis of the crude oil biodegradation that occurred during the incubation period showed that the communities from the MVs with different activities were better equipped to degrade different groups of hydrocarbons. Communities from active MV sediments were able to degrade *n*-alkanes from C_7 to C_{13}, with the last four only being degraded in the presence of nitrate, all of the monoaromatic hydrocarbons tested, and anthracene. Communities from inactive MV cultures degraded heptane and octane, toluene and six polycyclic aromatic hydrocarbons, flourene, phenanthrene, anthracene, pyrene and benzo[*b*]flouranthene plus benzo[*k*]flouranthene. Nitrate only had a positive effect in the biodegradation of C_{10} to C_{13} alkanes by active MV communities, either not affecting or having a negative impact in the biodegradation of other hydrocarbons. This work allowed to identify the main players in anaerobic PHs biodegradation in the deep-sea MVs from gulf of Cadiz as well as their degradative ability towards a mixture of PHs. Since the deep-sea is considered to be the last frontier of the planet, due to the lack of biological information, this work contributes to the characterization of bacterial communities in these environments and their reaction under different stimuli. This information may also contribute to the design of site-specific bioremediation strategies in case of PHs contamination of marine sediments and to better understand the metabolic capability of these communities in the context of hydrocarbon degradation. This data can also help predict how the bacterial communities from Gulf of Cadiz MVs would react to crude oil contamination. The irregular seepage of the MVs' mud coupled with consumption of the hydrocarbons seeping from the subsurface marine sediments often makes it difficult to ascertain the level of activity of MVs. Taxonomic groups increasing or appearing in the presence of crude oil may be used as biosensors to indicate the activity state of the MVs from a microbiological point-of-view.

In a second phase of the experimental work, anaerobic selective cultures were inoculated with subaquatic estuarine and deep-sea MVs sediments and designed to test the effect of crude oil, nitrate and sulfate amendments. Both sediment types were rich in NRB but sulfide-producing bacteria were only detected in cultures with estuarine sediments. Isolates were grown and purified in solid media, with only crude oil as carbon source, under anaerobic conditions. Of the 35 isolates presenting less than 90% similarity in BOX-PCR 13 were able to produce BSFs under aerobic conditions. Six were isolated from cultures with estuarine sediments and seven from cultures with MVs sediments. *Pseudomonas* was the most common genera, with two isolates from each environment. The second most common genera were Bacillus, originating from MVs sediments, and *Ochrobactrum,* from estuarine sediments. Other isolates belonged to genera *Brevundimonas, Psychrobacter*, *Staphylococcus*, *Marinobacter* and *Curtobacterium* (with one representative each) with the first three being isolated from MVs cultures and the last two from estuary cultures. All of these isolates were able to grow with only crude oil as carbon source and eleven of the isolates were

able to produce BSFs under anaerobiosis. Four isolates, belonging to the genera *Psychrobacter, Curtobacterium* and *Pseudomonas* (2), were also able to produce molecules with high emulsification ability under aerobic conditions and one of the Pseudomonas was also able to do so in anaerobiosis. A previously isolated and identified *Pseudomonas* sp. 74 was also characterized as being able to produce BSF under anaerobic conditions. So far no other known anaerobic BSF-producing bacteria had been reported as belonging to the *Ochrobactrum, Psychrobacter, Staphylococcus, Brevundimonas* and *Marinobacter* genera. Likewise, no known BSF-producing bacteria had yet been described as belonging to the *Curtobacterium* genus, independently of the oxygen condition. Therefore, this work led to a significant increase in the knowledge of new hydrocarbonoclastic BSF-producers overall. More specifically it increased the pool of known BSF-producers under oxygen-limiting conditions.

5.1. Future work

Despite the results presented here further work is required to apply the knowledge gained in biotechnological applications.

Some progresses are being made towards a better understanding of the metabolic pathways involved in aerobic hydrocarbon degradation and BSF production. However, particularly for the latter, very little is known about the metabolic pathways used in the absence of oxygen. Further studies towards a better understanding of anaerobic metabolic pathways involved in these processes are fundamental, to better predict how bacterial biodegradation will change and affect petroleum composition and how the lack of oxygen can affect BSF composition, possibly leading to an increase of known BSFs.

The optimization of culture media is fundamental to increase BSF production and in some cases, determine BSF composition. Therefore, this should be the next step in the exploration of the biotechnological potential of the isolates obtained in the present work. Utilization of industrial by-products should also be considered as a low-cost carbon source alternative.

BSF extraction is also a crucial step. However, since the success of the extraction strategies available depend on the BSF, and most protocols are designed for rhamnolipids and surfactin, it is possible that this step may represent a considerable challenge for genera producing unknown BSFs. Following extraction, the BSFs should be identified, by techniques such as chromatography and mass spectroscopy, and characterized, by determining its critic micellar concentration (CMC) and stability in different pHs, temperatures and salinities. BSFs may also have antimicrobial properties, which are of particular interest in pharmaceutical and food industries, and therefore should be tested for this as well.

Only after these steps can the isolates be considered for biotechnological applications, depending on their characteristics. Bacteria producing novel BSFs with either low CMCs, high E_{24} or antimicrobial properties can be of interest for industrial production of BSFs. Bacteria able degrade hydrocarbons and produce BSFs under various degrees of oxygen concentration can be used in MEOR or bioremediation strategies.

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